Interaction of Transforming Growth Factor α with the Epidermal Growth Factor Receptor: Binding Kinetics and Differential Mobility within the Bound TGF-α†

David W. Hoyt,‡ Richard N. Harkins,§ Maria T. Debanne,|| Maureen O'Connor-McCourt,|| and Brian D. Sykes*,‡

Protein Engineering Network of Centres of Excellence and the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada, Berlex Biosciences, Richmond, California 94804, USA, and Biotechnology Research Institute, Montreal, Quebec H4P 2R2, Canada

Received June 16, 1994; Revised Manuscript Received September 8, 1994®

ABSTRACT: The interaction of transforming growth factor α (TGF-α) with the complete extracellular domain of the epidermal growth factor receptor (EGFR-ED) was examined by nuclear magnetic resonance (NMR) spectroscopy. The ^{1}H NMR resonances of the methyl groups of TGF-α were used as probes of the interaction of TGF-α with the EGF receptor to determine the binding kinetics and the differential mobility within the bound TGF-α. The methyl resonances were studied because there are 14 methyl containing residues well dispersed throughout the structure of TGF-α and the relaxation properties of methyl groups are well understood. Changes in the longitudinal and transverse ^{1}H NMR relaxation rates of the methyl resonances of TGF-α caused by binding to the 85-kDa EGFR-ED were studied. From these measurements it was determined that the interaction was in the NMR fast exchange limit. A binding mechanism to rationalize the different rates determined by NMR and surface plasmon resonance techniques [Zhou, M., et al. (1993) *Biochemistry 32*, 8193−8198] is proposed. The transverse relaxation rate (R_2) enhancements of the various methyl resonances displayed a regional dependence within the bound TGF-α molecule. Resonances from the C-terminus of TGF-α, which were flexible in the unbound molecule, revealed dramatic increases in their R_2 upon binding to the EGFR-ED along with resonances from the interior of TGF-α. However, upon binding, the R_2 enhancements of the methyl resonances from the N-terminus of TGF-α, which were also flexible in the unbound TGF-α, were slight; indicating a retention of mobility of this region for bound TGF-α. The implications of these data with respect to the mechanism of receptor activation and the design of antagonists are discussed.

The formation of the growth factor/receptor complex is the pivotal first step to triggering the growth response in cells. The well characterized epidermal growth factor (EGF), when bound to the 170-kDa epidermal growth factor receptor (EGFR), is responsible for signaling the normal cellular growth of epithelial and mesenchymal cells [for reviews, see Carpenter (1987), Carpenter and Cohen (1990), and Staros et al. (1989)]. Human transforming growth factor α (TGF-α)[1] has a similar binding affinity for EGFR and has no other known receptor (Lax et al., 1991; Korc & Finman, 1989; Carpenter, 1987). Involvement in embryonic development of hair and epithelial tissues and wound healing processes are cited as possible biological roles for TGF-α (Mann et al., 1993; Luetteke et al., 1993; Martin et al., 1992; Dougall et al., 1993). Yet the major biological interest in TGF-α comes from studies implicating this growth factor in the transformation and maintenance of the growth of several different types of malignant tumors (including breast, renal, and squamous cancers) (Derynck et al., 1987; ten Dijke et al., 1989).

In addition to binding to the same receptor, the 50-residue human-TGF-α shares 40% sequence identity with the 53-residue human-EGF. Further, both proteins are divided into three loops (A, B, and C) by three conserved disulfide bonds. Over the past decade, various attempts have been made to define the functionally important domains and residues within EGF and TGF-α. These include recombinant DNA techniques to identify functional residues (Defeo-Jones et al., 1988, 1989; Lazar et al., 1988, 1989; Engler et al., 1988, 1991; Matsunanmi et al., 1991), monoclonal antibodies studies to identify important peptide segments (Katsuura & Tanaka, 1989; Hoeprich et al., 1989; Richter et al., 1992), and the mitogenic activity of synthetic peptides derived from EGF and TGF-α (Heath & Merrifield, 1986; Darlak et al., 1988; Eppstein et al., 1989; Tam et al., 1991). These studies have implicated certain residues in TGF-α such as R42 and L48 (or R41 and L47 in EGF) as critical for function (Engler et al., 1990; Matsunanmi et al., 1991) and have suggested that large portions of more than one loop are required for mitogenic activity (Tam et al., 1991; Richter et al., 1992).

† This project was support by the Government of Canada through the Networks of Centres of Excellence Program and by Berlex Biosciences, Inc., USA.

* To whom correspondence should be addressed at PENCE, 7-13 Heritage Medical Research Centre, University of Alberta, Edmonton, AB T6G 2S2, Canada. Phone: (403) 492-6540. Fax: (403) 492-1473. E-mail: bds@polaris.biochem.ualberta.ca.

‡ University of Alberta.

§ Berlex Biosciences.

|| Biotechnology Research Institute.

® Abstract published in *Advance ACS Abstracts,* November 15, 1994.

[1] Abbreviations: NMR, nuclear magnetic resonance; CPMG, Carr-Purcell−Meiboom-Gill pulse sequence for measuring transverse relaxation rates; TGF-α, human transforming growth factor α ($M_r \gg 5800$); EGFR-ED, baculovirus-expressed human epidermal growth factor receptor extracellular domain ($M_r \gg 85\,000$); NOE, nuclear Overhauser effect; R_1, longitudinal relaxation rate constant; R_2, transverse relaxation rate constant; T_1, longitudinal relaxation time; T_2, transverse relaxation time; NOESY, 2D NOE spectroscopy; TOCSY, 2D total correlation spectroscopy; DSS, sodium-2,2-dimethyl-2-silapentane-5-sulfonate.

0006-2960/94/0433-15283$04.50/0

However, a clear consensus of the requirements for activity and binding of these growth factors to EGFR has not been established.

Structural studies of EGF and TGF-α have been pursued vigorously in hopes of gaining knowledge of the important structural features necessary for binding and activation of the receptor. To date, the vast majority of the structural work has been centered on the structure of the growth factors themselves in the absence of the receptor, and relatively little work has been focused on the growth factor−receptor complex (Rousseau et al., 1993; Zhou et al., 1993). The only structure for a growth factor−receptor complex known is the X-ray crystal structure of human growth hormone−human growth hormone binding protein complex (deVos et al., 1992). Crystallographic studies have been unsuccessful in yielding structures of any EGF-like growth factor receptor complexes. However, X-ray studies have yielded the structure of the EGF-like domain of human factor X_a, (Padmanabhan et al., 1993) and high-resolution ^{1}H 2-D NMR studies have yielded the detailed tertiary structures of human EGF, murine EGF, and human TGF-α in solution (Cooke et al., 1987; Kohda et al., 1989; Kline et al., 1990; Harvey et al., 1991; Montelione et al., 1992) as well as the EGF-like factor IX (Baron et al., 1992). More recently, heteronuclear 3-D NMR experiments have resulted in higher resolution structures of human EGF and human TGF-α (Hommel et al., 1992; Moy et al., 1993).

While high-resolution multidimensional NMR methods have been successful in determining the solution structures of EGF and TGF-α, the determination of the structure of the complex of TGF-α with the membrane-bound EGF receptor is clearly well beyond the limits of NMR methods. Even if the water-soluble extracellular domain of the EGF receptor (EGFR-ED) is used, the total molecular weight of TGF-α (6 kDa) and EGFR-ED (85 kDa) is more than twice the MW limit for the applicability of three- and four-dimensional multinuclear NMR methods. The largest complex to date to have its structure determined in solution by NMR is that of calmodulin-myosin light chain kinase peptide (MW ≫ 20 000) (Ikura et al., 1992). However, for many applications such as the design of small molecule antagonists, it is sufficient to characterize the structure, dynamics, and environment of the bound ligand. Over the last 30 years a large variety of NMR techniques have been used to study the interaction of ligands with large proteins (Sykes & Scott, 1972; Dwek, 1973; Jardetzky & Roberts, 1981). This includes the recent resurgence of techniques such as the transferred NOE to determine the structure of bound ligands (Campbell & Sykes, 1993; Sykes, 1993; Anglister & Naider, 1991) and the use of multinuclear multidimensional NMR to determine the bound structure of ligands such as cyclosporin bound to the target protein cyclophilin (Fesik et al., 1991). The selection and applicability of the various possible NMR approaches depends upon a variety of factors such as the affinity for the ligand and the exchange rate of the ligand on and off of the receptor biomolecule.

The focus of this paper is to use NMR relaxation measurements to study TGF-α in the presence of the extracellular domain of the EGF receptor to characterize both the chemical exchange of TGF-α on and off of the receptor and the dynamic properties of TGF-α when bound to the EGF receptor. In particular, while many recent studies in other systems have used ^{13}C and ^{15}N NMR relaxation measurements to characterize molecular motion (Palmer et al., 1993; Cheng et al., 1993, 1994; Grasberger et al., 1993), we have chosen to study the ^{1}H NMR relaxation behavior of the methyl residues of TGF-α to probe the regions of TGF-α which interact with the receptor. We present a detailed description of the experimental procedures, the evidence which discriminates the regions of TGF-α that have restricted mobility upon binding from those that do not, the limits on the exchange rates, and a discussion of the implications of these results for a possible mechanism by which TGF-α and EGF may bind and activate EGFR.

MATERIALS AND METHODS

Expression and Purification of TGF-α. Recombinant human TGF-α was expressed in *Escherichia coli* (expression plasmid PTBI102-trpE/TGF) as a trpE fusion protein (first 15 amino acids of the E gene of the tryptophan operon plus Glu-Phe-Met plus 50 amino acids of TGF-α) under control of the bacteriophage lambda promoter pL in the prototroph strain ETB-64(W1485). The fermentation for the expression of ^{15}N-enriched TGF-α was performed by growing the bacteria on a modified minimal media [variation of M63 Medium described by Miller (1972)]. Expression of the TGF-α fusion protein was induced by a 32−42 °C temperature shift during the fermentation run (3.5 h, 3.5 OD_{550}). Cells were harvested and stored at −70 °C.

Cells were lysed by sonication and then centrifuged. The TGF-α fusion protein is found in the pellet fraction as inclusion bodies. The protein is solubilized by extraction of the inclusion bodies in 6 M guanidine-HCl, containing 0.1% trifluoroacetic acid. The fusion protein was treated with cyanogen bromide to remove the 18-residue trpE leader. Mature TGF-α was purified by preparative HPLC. Refolding of TGF-α was performed by sulfitolysis (Spear & Sliwkowski, 1991) followed by a final purification on a Mono-S column (Pharmicia). The eluted fractions containing the TGF-α were lyophilized for the preparation of NMR samples.

Purified, refolded, TGF-α was shown to be homogeneous based upon NH_2-terminal sequence analysis and was assessed to be greater than 98% pure by reverse-phase analytical HPLC using a C-18 column (SynChropak RP-PC_{18}, 250 × 4.6 mm id, 300-Å pore size, SynChrom, Inc., Layfayette, Indiana). The correct primary ion peaks of the expected molecular mass of 5544 Da was confirmed for TGF-α by a BIOION-20 Nordic plasma-desorption time-of-flight mass spectrometer (Uppsala, Sweden). In addition, the protein was also shown to be fully active in an EGF radioreceptor binding assay using membranes enriched in the EGFR from human A431 cells. This assay is based upon the ability of TGF-α to compete with ^{125}I-EGF for binding to the EGFR.

Expression, Large Scale Production, and Purification of the EGFR-ED. Recombinant baculovirus carrying the EGFR-ED cDNA was generated as previously described (Brown et al., 1994). Ten liters of Sf9 cells (5−10 × 10^6 cells/mL) grown in suspension culture [SF400 media (Gibco)] was infected with recombinant baculovirus at an MOI of between 0.5 and 3. The culture supernatant was harvested at 72 h postinfection. A protease inhibitor (0.1 mM PMSF) and glycerol (10% v/v) were added, and the media was stored at −20 °C until purification. Purification of the EGFR-ED involved a mild, nonaffinity, two-step ion exchange chromatography procedure (Debanne et al., 1994). The EGFR-

ED was quantitated during purification according to an immunoenzymetric assay (ELISA) (Grimaux et al., 1989). The protein concentration of the purified receptor was determined by Lowry. The degree of purity was 85% or greater as determined by densitometric scanning of Coomassie Blue or silver-stained SDS−PAGE gels. The percentage of active EGFR-ED in the preparations (90% or greater) was determined using a band shift technique (Debanne et al., 1994).

NMR Sample Preparation. Lyophilized TGF-α samples were dissolved into 460 μL of buffer containing 50 mM potassium phosphate, 10 mM potassium chloride, 1 mM ethylene diamine tetraacetic acid, 0.5 mM sodium azide, 0.15 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, internal standard), and 99.9% D_2O or 90%/10% (v/v) H_2O/D_2O. The solution was adjusted to pH 6.0 or 8.0 by the addition of microliter amounts of 0.5 N NaOD or 0.5 N HCl bringing the final volume to 500 μL (measurements uncorrected for deuterium isotope effects). Protein concentrations are indicated in the figure legends.

Stock solutions of EGFR-ED were made by dialyzing the sample against the phosphate buffer used above to prepare TGF-α. The receptor solutions were 200 μM of active EGFR-ED in 500 μL.

NMR Measurements. NMR experiments were recorded at 298 K using a Varian Unity-600 NMR spectrometer operating at 599.9 MHz. All 1-D and ^1H relaxation spectra were acquired with a $^1H-^{13}C-^{15}N$ triple-resonance probe; some assignment spectra were recorded using an indirect detection probe. For all experiments, the spectral width in the ^1H dimension(s) was 8000 Hz. The ^1H chemical shift is relative to an internal DSS standard. The ^1H carrier frequency was placed on the H_2O peak (4.78 ppm).

For 2-D homonuclear phase-sensitive DQF-COSY (Piatini et al., 1982; Rance et al., 1983), TOCSY (Bax & Davis, 1985; Griesinger et al., 1988), and NOESY (Bodenhausen et al., 1984; Kumar et al., 1980; Jeener et al., 1979) spectra, 256 or 320 complex t_1 experiments (48 or 64 signal averaged transients) were recorded consisting of 1024 complex points in t_2. Quadrature detection was accomplished using the hypercomplex method (States et al., 1982). Mixing times (t_m) of 30 or 50 ms were used for TOCSY spectra and 50, 100, and 200 ms were used for the NOESY spectra. During the NOESY experiments, solvent peak recovery was reduced by the inclusion of a 180° composite pulse in the center of the mixing period (Brown et al., 1988). In all other experiments the H_2O peak was suppressed by low-power coherent irradiation of the water resonance for 1.0−2.0 s prior to each pulse train.

Spin−lattice relaxation times T_1 were measured using the standard inversion−recovery pulse sequence (Vold et al., 1968). Transverse spin−spin relaxation times T_2 were measured using the Meiboom-Gill modification of the Carr-Purcell experiment (Meiboom-Gill, 1958). In these experiments the time delay between 180° pulses was kept small ($2\tau = 0.00025$ s) to minimize dephasing due to spin−spin coupling. The relaxation curves were fit to $M_z(t) = M_z(0)[1 - w \exp(-t/T_1)]$ and $M_{xy}(t) = [M_{xy}(0) \exp(-t/T_2)]$ for T_1 and T_2 measurements, respectively, where w is the 180° pulse inversion factor ($w = 2$ for perfect 180° pulse). Values obtained for w were typically 1.8−1.9.

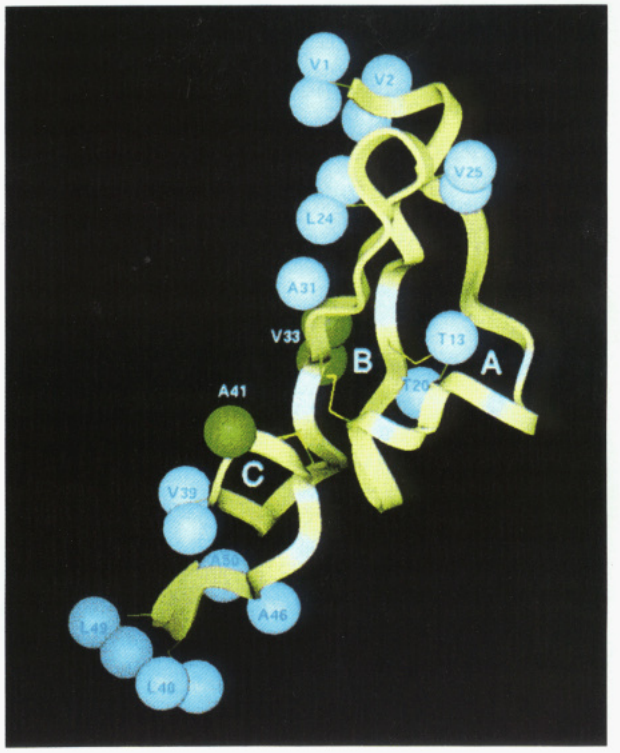

FIGURE 1: Ribbon diagram of the TGF-α structure with CPK depiction of the methyl groups. The structure is the RMSD average of published NMR data (Harvey et al., 1991) obtained from the Brookhaven Data Bank. The ribbon (gold) traces the peptide backbone atoms of TGF-α. The side chain methyl groups from residues Val 1, Val 2, Thr 13, Thr 20, Leu 24, Val 25, Ala 31, Val 39, Ala 46, Leu 48, Leu 49, and Ala 50 which were analyzed extensively in this paper are labeled and appear in white. The side chain methyls of Val 33 and Ala 41 appear in green. The three disulfide bridges between residues 8−21, 16−32, and 34−43 are shown in yellow, and the resulting A, B, and C "loops" are labeled.

RESULTS

Methyl Resonances as Molecular Probes. There are several reasons for choosing the ^1H NMR relaxation behavior of the methyl residues of TGF-α to probe the chemical exchange of TGF-α with the water soluble extracellular domain of the EGFR receptor and to determine the dynamic properties of TGF-α when bound to the receptor. From the structural standpoint, human TGF-α has 14 residues out of 50 which contain a total of 22 methyl groups. These methyl-containing residues are punctuated in the primary sequence and are well-spaced throughout the TGF-α structure (Figure 1). In Figure 1, the distribution of the methyl groups is seen in CPK form against the ribbon through the backbone atoms. The residues containing methyl groups which were analyzed most extensively are colored white while the remaining methyl groups are colored green. The methyl groups of TGF-α are excellent probes for NMR relaxation measurements since their resonances are well resolved, have been completely assigned, have an intensity three times that of a single proton resonance, have narrow line widths because of internal motion (see below), and have a simple NMR coupling pattern which leads to easily identifiable doublets. From a relaxation standpoint, the methyl protons are suitable because the ^1H NMR relaxation of geminal or methyl protons is dominated by the dipole−dipole relaxation involving geminal protons separated by a fixed internuclear distance, and the internal motion of methyl groups is simple and well understood (Marshall et al., 1972; Sykes et al., 1978;

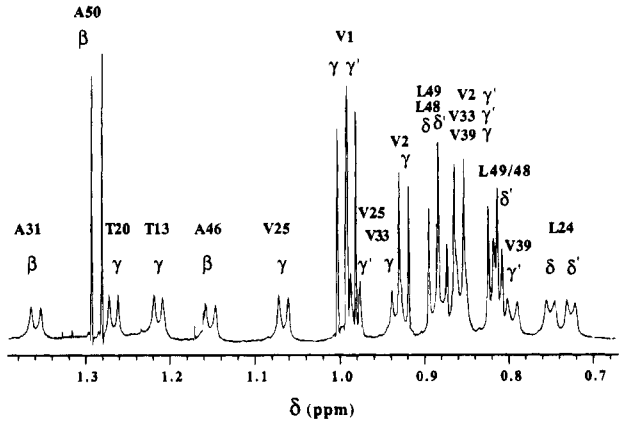

FIGURE 2: Methyl group region of the 1-D ^{1}H NMR spectrum of TGF-α. The protein concentration was 2 mM, and the solution was buffered at pH 6.0. Spectrum was recorded at 298 K and at 600 MHz. This spectrum represents 512 scans with a digital resolution of 0.25 Hz/pt (32K data points collected). Processing was accomplished using resolution enhancement with window functions of −1.60 = lb and 0.60 = gf.

Goldman, 1988).[2] The ^{1}H NMR relaxation of methyl protons relaxing via the dipole−dipole mechanism where the contributions of internal motion are not yet considered is given by

$$R_2 \equiv 1/T_2 = A\{3\tau_c + [5\tau_c/1 + (\omega_o\tau_c)^2] + [2\tau_c/1 + (2\omega_o\tau_c)^2]\}$$

$$R_1 \equiv 1/T_1 = 2A\{[\tau_c/1 + (\omega_o\tau_c)^2] + [4\tau_c/1 + (2\omega_o\tau_c)^2]\} \quad (1)$$

Where $A = (3/20)(N-1)(\gamma^4h^2/r^6) = 5.0 \times 10^9\ s^{-2}$ for N = three equivalent spins separated by $r = 1.8$ Å, ω_o is the NMR resonance frequency, and τ_c is the correlation time for the internuclear vector between the methyl protons.

Presented in Figure 2 is a 1-D ^{1}H NMR spectra of TGF-α. The spectral region shown is the upfield-shifted region which contains only methyl proton resonances (except A41 which appears at 1.57 ppm, is not well resolved from other sidechain resonances, and is not discussed further). The methyl resonances have all been assigned using standard 2-D and 3-D NMR methods under the conditions used in this study (see Table 1) and most are well resolved. The most striking feature are the differential line widths which must result from different correlation times for the various methyl-containing residues in TGF-α. These differences in correlation times are caused by differences in motion for the various residues. Motions of the protein as a whole as well as internal dynamics of the backbone and side chains must be considered. To understand differential line width, we have to first consider the effects of internal motion of the methyl group. The effects of line narrowing on a ^{1}H resonance of equivalent protons via internal mobility can be corrected under conditions where $\tau_{int} \ll \tau_c$; yielding an effective correlation time τ_c^{eff} (Marshall et al., 1972):

[2] In formal terms the relaxation of AX_3 and similar spin systems can be quite complex due to effects such as cross-correlation (Werbelow et al., 1977), especially in the presence of anisotropic overall motion; in practice, the observable effects in terms of nonexponential relaxation behavior are small (Werbelow & Marshall, 1974) and are neglected herein.

Table 1: ^{1}H NMR Assignments TGF-α at pH 6.0, 25 °C[a]

	chemical shift (ppm)			
residue	H_N	H_α	H_β	other protons
Val 1		3.87	2.18	H_3^γ 1.00, 0.99
Val 2	8.55	4.16	2.01	H_3^γ 0.92, 0.86
Ser 3	8.48	4.41	3.72, 3.72	
His 4	8.47	4.67	3.12, 3.00	H^2 8.37, H^4 7.09
Phe 5	8.26	4.98	3.12, 2.94	$H^{2,6}$ 7.17; $H^{3,5}$ 7.31
Asn 6	8.74	4.94	2.92, 2.73	$H^\delta N$ 7.62, 6.94
Asp 7	8.22	4.66	2.68, 2.52	
Cys 8	8.92	4.31	3.12, 3.12	
Pro 9		4.41	2.18, 1.95	H^γ 1.68, 1.68; H^δ 3.23, 3.13
Asp 10	8.55	4.42	2.68, 2.68	
Ser 11	8.11	4.26	3.78, 3.78	
His 12	8.39	4.73	3.43, 2.89	H^2 8.06, H^4 6.72
Thr 13	7.77	4.18	4.14	H^γ 1.21
Gln 14	8.43	4.31	2.03, 1.78	H^γ 2.16, 2.16; $H^\epsilon N$ 7.37, 6.77
Phe 15	7.94	4.16	3.10, 2.87	$H^{2,6}$ 7.12; $H^{3,5}$ 7.19
Cys 16	8.28	4.34	2.49, 2.03	
Phe 17	8.30	4.22	2.71, 2.71	$H^{2,6}$ 7.09; $H^{3,5}$ 7.12
His 18	6.81	4.48	2.72, 2.28	H^2 8.42, H^4 6.89
Gly 19	7.40	3.89, 3.58		
Thr 20	8.43	4.49	3.92	H^γ 1.27
Cys 21	9.05	5.17	3.23, 3.22	
Arg 22	9.44	4.74	1.64, 1.58	H^γ 1.45, 1.45; H^δ 2.82, 2.82
Phe 23	9.07	4.63	2.92, 2.70	$H^{2,6}$ 6.85; $H^{3,5}$ 7.11
Leu 24	8.27	4.46	1.74, 1.51	H^γ 1.51; H_3^δ 0.75, 0.73
Val 25	8.04	3.68	2.03	H_3^γ 1.07, 0.98
Gln 26	8.82	4.09	2.08, 2.08	H^γ 2.40, 2.40; $H^\epsilon N$ 7.63, 6.85
Glu 27	7.74	4.18	1.70, 1.56	H^γ 2.18, 2.05
Asp 28	7.94	4.26	3.09, 2.38	
Lys 29	6.94	4.66	1.72, 1.55	H^γ 1.23, 1.23; H^δ 1.65, 1.57; H^ϵ 2.82, 2.82
Pro 30		4.68	1.74, 1.59	H^γ 1.33, 1.33; H^δ 3.48, 3.36
Ala 31	8.88	4.73	1.36	
Cys 32	8.74	5.29	2.78, 2.61	
Val 33	8.99	4.23	2.00	H_3^γ 0.93, 0.86
Cys 34	9.14	5.04	3.44, 2.74	
His 35	8.70	4.86	3.40, 2.90	H^2 7.90; H^4 7.06
Ser 36	8.66	4.27	3.92, 3.92	
Gly 37	8.83	4.16, 3.58		
Tyr 38	8.01	5.26	2.92, 2.76	$H^{2,6}$ 6.67; $H^{3,5}$ 6.40
Val 39	9.20	4.85	2.27	H_3^γ 0.86, 0.80
Gly 40	8.12	5.04, 3.90		
Ala 41	9.24	4.17	1.57	
Arg 42	8.75	4.73	2.28, 2.28	H^γ 1.38, 0.84; H^δ 2.93, 2.93
Cys 43	8.10	3.98	2.94, 2.48	
Glu 44	10.02	4.26	1.64, 1.64	H^γ 1.87, 1.45
His 45	8.57	5.15	3.14, 3.05	H^2 8.38, H^4 7.12
Ala 46	8.74	4.18	1.16	
Asp 47	8.11	4.43	2.49, 2.21	
Leu 48	8.08	4.25	1.58, 1.58	H^γ 1.58; H_3^δ 0.88, 0.81
Leu 49	8.19	4.31	1.64, 1.64	H^γ 1.58; H_3^δ 0.89, 0.82
Ala 50	7.61	4.06	1.29	

[a] The ^{1}H chemical shift values (±0.02 ppm) were derived from the 2D ^{1}H TOCSY NMR spectrum. The ^{1}H chemical shift is relative to an internal DSS standard. Assignments were made using standard sequential assignment methods including 3-D ^{15}N-edited NOESY-HMQC and 3-D ^{15}N-edited TOCSY-HMQC NMR spectra.

$$\tau_c^{eff} = [(1 - 3\cos^2\theta)/2]^2\tau_c \quad (2)$$

The $\tau_{int} \ll \tau_c$ assumption is met safely since the internal rotation ($\tau_{int} \approx 10^{-11}-10^{-12}$ s) of methyl groups is expected to be several orders of magnitude faster than the overall tumbling of the 6-kDa TGF-α ($\tau_c \approx 2 \times 10^{-9}$ s) especially when bound to the 85-kDa EGFR-ED [$\tau_c \approx 60 \times 10^{-9}$ s, estimated from the Stokes−Einstein equation; see Brauer and Sykes (1987)]. For CH_3 groups, $\theta = 90°$ (measured as the angle between the H−H internuclear vector and the C3 axis of internal motion), and thus $\tau_c^{eff} \cong {}^1/_4\tau_c$. Furthermore, this narrowing effect due to internal methyl rotation on line width

Table 2: Relaxation Rates for TGF-α Methyl Resonances as a Function of Added EGFR-ED

[TGFα]/[EGFRx]	P_B[a]	V1	V2	T13	T20	L24	V25	A31	V33	V39	A46	L48/49	A50
						(A) Longitudinal Relaxation Rates (R_1) (s^{-1})							
	0.00	1.8	1.7	2.0	2.3	2.0	1.6	2.2	1.7	2.1	1.9	1.6	1.4
10.0	0.10	2.0	2.2	3.1	3.0	3.0	2.3	3.5		3.6	3.1	2.5	2.0
7.5	0.13	2.1	2.3	3.3		3.3	2.5	3.9			3.2	2.6	
5.0	0.20	2.2	2.3	3.7		3.7	3.0	3.8			3.3	2.9	
3.5	0.29	2.3	2.4			4.4						3.2	
2.0	0.50	2.4	2.4										
						(B) Transverse Relaxation Rates (R_2) (s^{-1})							
	0.00	4	6	19	13	10	11	30	14	16	33	5	5
10.0	0.10	6	10	34	25	23	30	34		60	62	25	23
7.5	0.13	6	10	36		27	30	42			72	29	
5.0	0.20	6	11	49		40	37	43				50	
3.5	0.29	7	11			66						90	
2.0	0.50	8	11										

[a] $P_B = [EGFRx{\cdot}TGF\alpha]/[TGF\alpha]_{total} \approx [EGFRx]_{total}/[TGF\alpha]_{total}$. This approximation assumes the receptor is saturated. This assumption is valid if [TGFα], [EGFRx] $\gg K_D$ and is met with the experimental conditions used (millimolar components and micromolar K_D).

of all methyl proton resonances will be equal. Therefore the differential line width seen in Figure 2 between methyl resonances must be a result of differences in motion in the peptide backbone or in the side chain of the longer chain residues such as Val and Leu. The very sharp resonances are from methyl-containing residues in the N and C termini such as V1 and V2, and L48, L49, and A50.

The observed differential line widths for TGF-α in the absence of receptor are supported by T_1 and T_2 relaxation time measurements for each methyl resonance. The values are reported as relaxation rate constants R_1 and R_2, where the R_2 values are directly proportional to line width ($\Delta\nu = R_2/\pi$). Therefore, residues with the smallest R_2 values correspond to resonances with the narrowest lines. These relaxation rate constants for the free TGF-α are summarized for each methyl-containing amino acid residue in the first row of Table 2, sections A and B, respectively. For leucine or valine residues, only one value is listed since the relaxation rates of both methyl resonances were found to be identical. Comparing Table 2 sections A and B, it is observed that $R_1 < R_2$ in all cases. This indicates that even for free TGF-α the NMR relaxation is outside of the extreme narrowing limit [$(\omega_o\tau_c)^2 \approx 50 \gg 1$]. In this slow tumbling limit, and especially for the bound TGF-α, R_1 measurements will be dominated by cross-relaxation and uniform methyl internal motion (Sykes et al., 1978) and therefore are not useful probes of overall motion, whereas R_2 measurements will be directly proportional to τ_c,

$$R_2 = \frac{3}{4}A\tau_c \qquad (3)$$

and hence are good probes of differential internal motion. From the first row in Table 2A, the R_1 values span a narrow range of 1.4−2.3 s^{-1} with an average value of 1.9 s^{-1}. Therefore T_1 relaxation is approximately the same for all resonances in the free TGF-α as predicted.

In contrast, from the first row in Table 2B, the R_2 values cover a much larger range from 4 to 33 s^{-1}. The smallest R_2 values are seen for N-terminal residues V1 and V2 as well as the C-terminal residues L48/49 (which are not well resolved from each other) and A50. The largest values of R_2 are seen for A31 and A46. These residues have no additional internal motion other than methyl rotation and therefore may be more motionally restricted than other methyl-containing residues. Richardson and co-workers have encouraged the view that the C_β of alanines should be considered part of the backbone (Richardson et al., 1992). Thus it would not be surprising that the largest R_2 values correspond to the alanines. Although A46 is only four residues away from the flexible C-terminus, it is known to exist in a short β-structure (Harvey et al., 1991; Moy et al., 1993). This accounts for the pronounced difference in R_2 values between A46 and A50. Most of the methyl resonances fall into the intermediate range R_2 values. L24 which has two extra possible modes of internal rotation (α-β and β-γ), which may account for a smaller transverse relaxation rate of 10 s^{-1}. Having one extra possible rotation (α-β), threonine and valine residues display intermediate values with an average R_2 value of 15 s^{-1}. Together all these R_2 values give credence to the differential mobility of methyl-containing residues seen in the observed line widths of the methyl resonances in Figure 2 and suggest a model for free TGF-α of a relatively rigid core with quite flexible N- and C-termini.

TGF-α in the Presence of EGFR-ED. Initially, the first spectra examined of the TGF-α/EGFR-ED system were a series of 1-D spectra. In Figure 3 are 1-D ^1H NMR spectra of the titration of EGFR-ED with TGF-α. The top spectrum is of free TGF-α in the absence of EGFR-ED, and the assignments are indicated on this spectrum. The bottom spectrum is of EGFR-ED alone in the absence of TGF-α. Even though the MW of EGFR-ED is large, resonances can be seen in the 1-D NMR spectrum. Most of these are quite broad, although some relatively narrow methyl resonances near 0.8−1.0 ppm can be seen. The titration is over seven ratios of ligand to receptor (10:1 to 0.5:1) with the maximum concentration for TGF-α and EGFR-ED of 2 mM and 200 μM, respectively. It is clear from this figure that all TGF-α resonances are broadened in the presence of receptor but the increase in line width is not uniform. The methyl proton resonances from the C-terminal residues such as A50, L49, L48, and A46 are broadened more severely in the presence of receptor at lower ratios of TGF-α to EGFR-ED than the residues from other areas of the molecule; especially the N-terminal residues V1 and V2. Differential broadening of methyl resonance linewidths implies a significant change in the mobility of some residues but not others, but we must consider the effects of chemical exchange on the NMR relaxation measurements.

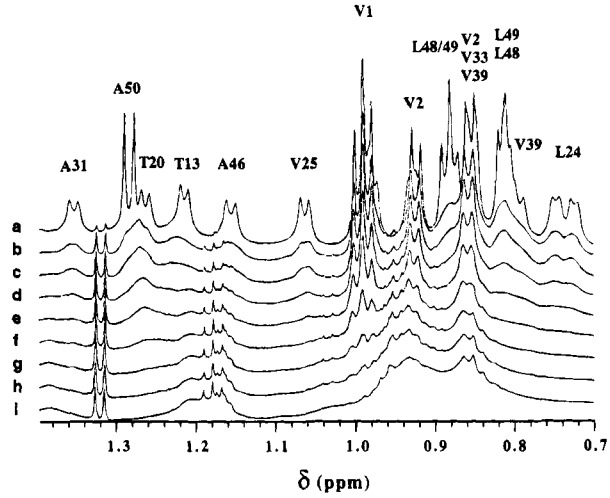

FIGURE 3: Observed 1-D ^{1}H NMR spectra of TGF-α titrated into EGFR-ED. The 1-D spectra shown are of EGFR-ED alone (bottom spectrum, i) with increasing mole ratios of TGF-α to EGFR-ED. The top spectrum (a) is of TGF-α alone and the assignments of the TGF-α methyl resonances are marked. The mole ratios of TGF-α to EGFR-ED of the indicated spectra are (b) 10:1, (c) 7.5:1, (d) 5:1, (e) 3.5:1, (f) 2:1, (g) 1:1, and (h) 0.5:1. Each spectrum is comprised of 512 scans with 0.25 Hz/pt digital resolution. Spectral processing was made with a 0.5 Hz line broadening. All spectra were recorded at 298 K and pH 6.0. Peaks at 1.18 ppm (triplet) and 1.32 ppm (doublet) are small molecule impurities.

In the presence of receptor, we have free and bound TGF-α and exchange between them

$$\text{TGF-}\alpha + \text{EGFR-ED} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{EGFR-ED·TGF-}\alpha \quad (4)$$

If we define the lifetime of the bound TGF-α as τ_B, the longitudinal and transverse ^{1}H NMR relaxation rates of the bound TGF-α as $1/T_{1B}$ and $1/T_{2B}$ of the free TGF-α as $1/T_{1F}$ and $1/T_{2F}$, and the fraction of bound and free TGF-α as P_B and P_F, then the observed relaxation rates can be written

$$R_{1obs} \equiv 1/T_{1obs} = P_F/T_{1F} + P_B/(T_{1B} + \tau_B)$$

$$R_{2obs} \equiv 1/T_{2obs} = P_F/T_{2F} + P_B/(T_{2B} + \tau_B) \quad (5)$$

This assumes there is no chemical shift difference between free and bound TGF-α protons as is shown to be the case (see Figure 3). Further we can define

$$R_{2p} \equiv 1/T_{2p} = 1/T_{2obs} - 1/T_{2F} = P_B[1/(T_{2B} + \tau_B) - 1/T_{2F}] \quad (6)$$

We examined the T_1 and T_2 relaxation of TGF-α in the presence of receptor for every point measured in the titration shown in Figure 3. Typical T_1 and T_2 relaxation measurements can be seen in Figure 4. Plots A and B are data extracted from T_1 and T_2 relaxation experiments (as peaks from panels C or E and D or F, respectively) curve fitted as described under Materials and Methods. The curve-fitting typically yields single-exponential curves. All values of T_1 and T_2 obtained are in Table 2 indicated in terms of relaxation rate constants R_1 and R_2.

In Figure 5, plots A and B graphically demonstrate the experimentally derived relaxation constants R_2 and R_1 as a function of the fraction of TGF-α bound to the EGFR-ED. The slope of these points are depicted for three representative

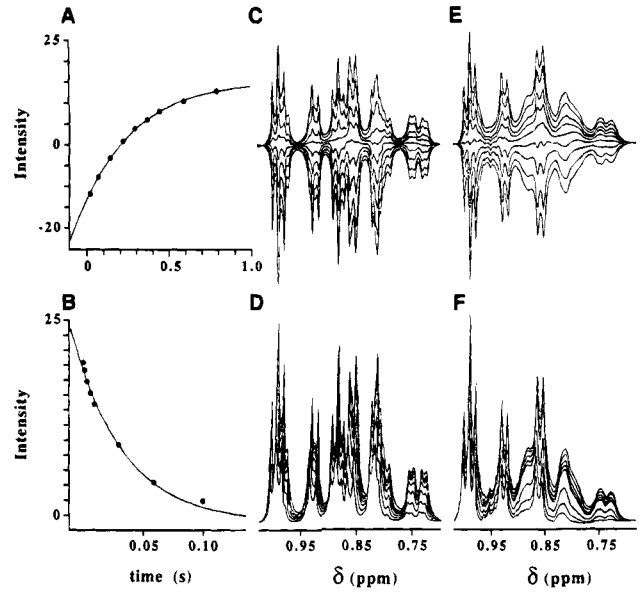

FIGURE 4: Typical T_1 and T_2 relaxation measurements for TGF-α. In panels A and B are plots of typical curve-fit data (specifically the methyl resonance of L24 at a TGF-α to EGFR-ED ratio of 10:1; raw data indicated in panels E and F) taken from T_1 and T_2 relaxation experiments, respectively. In panels C and E are spectra from T_1 relaxation measurements of TGF-α in the absence of receptor and at a 10:1 mol/mol ratio of TGF-α to EGFR-ED, respectively. Panels D and F are the analogous T_2 data. Fitted exponential increases from plots like panel A and exponential decays from plots like panel B yield longitudinal relaxation rate constants (R_1) and transverse relaxation rates (R_2), respectively, and these constants are reported in Table 2. The processing of the spectra shown was accomplished with a line broadening of 0.5 Hz.

residues V1, L24, and A46. The data for all the methyl-containing residues are given in Table 2. Plot C of Figure 5 displays the slope of R_{2p} versus fraction bound as a function of residue. These values are equivalent to the bound values for R_{2p}, designated R_{2p_max}. Figure 5B taken in comparison to Figure 5A (also refer to Table 2A,B) clearly shows that although the R_1 values for all resonances are increased somewhat in the presence of receptor, the values for each residue are approximately the same and the magnitude of the relaxation rates are much smaller than the contributions to R_2. This is as expected for the large molecular weight system (Sykes et al., 1978). Looking at Table 2B or Figure 5A,C, it should be noted that the R_2 values for each TGF-α methyl resonance increase at least modestly upon interaction with the EGFR-ED. However, the increases are not uniform as evidenced by their different slopes or bound line widths (Figure 5C).

A family of methyl resonances that are broadened by an average amount include T13, T20, and L24. Residues such as L48/L49 and A50 start out with high mobility (small R_2 values) and have a dramatic reduction in their mobility upon interaction with increasing amounts of receptor. Therefore the C-terminus appears to have lost its high flexibility upon binding to EGFR-ED. Residues, such as A46 and V39, start with large or intermediate R_2 values and undergo a large reduction in their mobility. Finally and most stunning are V1 and V2 which retain their high mobility upon binding to EGFR. These results together suggest that based on the protein dynamics of methyl-containing residues in TGF-α, it is possible to discriminate regions that do not participate in the binding of the ligand to receptor from those motionally

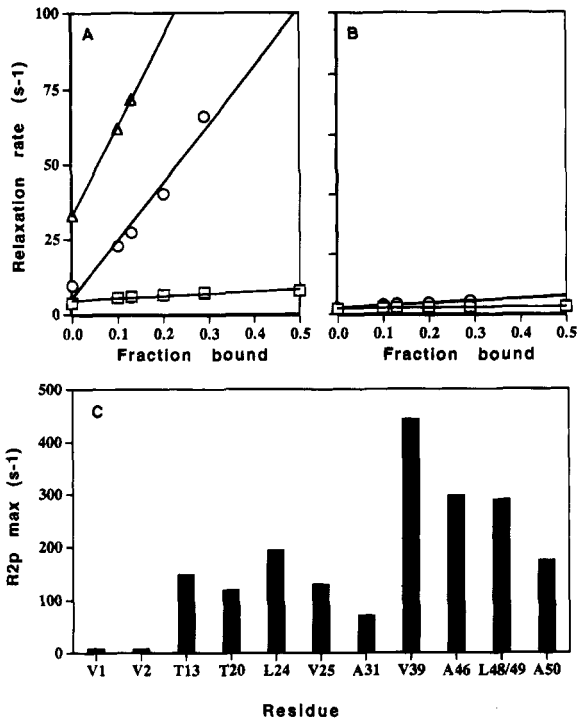

FIGURE 5: Comparison of the relaxation parameters of the methyl resonances of TGF-α. Plot A is a comparison of the transverse relaxation rates (R_2) of various methyl resonances as a function of mole fraction of bound TGF-α. Plot B compares the longitudinal relaxation rates (R_1) of TGF-α methyl resonances as a function of mole fraction of bound TGF-α. The data shown in plots A and B are of V1 (squares), L24 (circles), and A46 (triangles), and this subset is chosen to represent the trends and diversity of all the data. Plot C is a comparison of the slope of R_{2p} bound as a function of residue for the bound TGF-α for the whole data set.

restricted and therefore more intimately bound to the EGFR-ED.

Estimation of the Kinetic Parameters from Relaxation Parameters. Before the results presented in Figure 5 can be interpreted in terms of differential line widths and therefore differential mobility of bound TGF-α, the effects of chemical exchange on the relaxation time measurements must be understood. If the exchange rate (k_{off}) in eq 4 is small and the bound line widths large, then τ_B ($= 1/k_{off}$) will be larger than T_{2B} ($= 1/\Delta\nu_B$) and the relaxation enhancement R_{2p} would be the small and equal for all residues. This is clearly not observed (Figure 5A,C). Further, increasing temperature sharpens resonances slightly in the 1-D ^{1}H NMR spectrum of TGF-α in the presence of EGFR-ED (Figure 6), indicating that the binding is not in the slow exchange limit. Lifetime broadened line widths would be expected to increase in line width strongly with temperature as k_{off} increases with temperature. In the intermediate-to-fast exchange limit, the reduction in line width as a function of increasing temperature is due to the reduction of rotational correlation time ($\tau_c \propto \eta/T$). Thus τ_B must be $\leq T_{2B}$ (indicating fast exchange conditions) for all residues measured, and the most we can derive is a upper limit for τ_B derived from the resonances with the largest observed broadening. For V39 and A46, $R_{2p\_max} \ll 300-400$ s^{-1}, and thus $1/(T_{2B} + \tau_B) \ll 300-400$ s$^{-1} < 1/\tau_B$ so that $k_{off} > 300-400$ s^{-1}. If a diffusion-controlled k_{on} of 1×10^8 M^{-1} s^{-1} (Guttfreund, 1972) is assumed, then the expected K_D would be very approximately 3 μM.

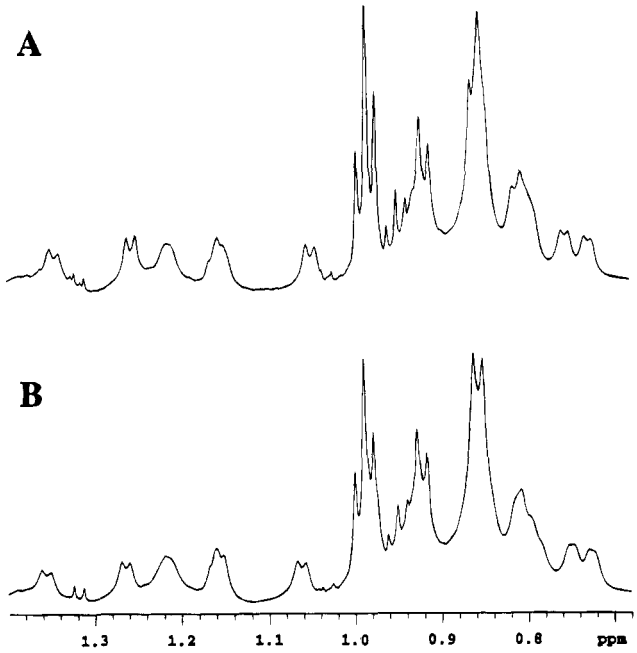

FIGURE 6: Comparison of the 1-D ^{1}H NMR spectrum of TGF-α in the presence of EGFR-ED (15:1) at 25 and 40 °C. The top spectra (A) is recorded at 40 °C and the bottom (B) at 25 °C. Each spectrum represents 512 scans with a digital resolution of 0.25 Hz/pt (32K data points collected). Spectral processing was made with a 0.5 Hz line broadening. Spectra were recorded at pH 6.0 and at 600 MHz. The TGF-α concentration 0.9 mM and EGFR-ED at 0.06 mM.

DISCUSSION

We have used ^{1}H NMR relaxation measurements, specifically of resonances containing methyl groups, to determine the kinetics of the interaction of TGF-α with the extracellular domain of the EGF receptor and the dynamics of TGF-α both when free in solution and when bound to the EGFR-ED. The goal of these measurements is to thereby determine which portions of TGF-α are in contact with the receptor and by implication important to the binding. These measurements have been done under conditions (pH 6) where the binding of TGF-α is weaker and the exchange of TGF-α between the free and bound states is rapid on the NMR relaxation time scale. The k_{off} rate was found to be >300−400 s^{-1}, which has allowed us to determine the relative line widths of this growth factor when bound to its receptor which would not have been possible for much tighter binding.

Schlessinger and co-workers (Zhou et al., 1993) have reported a K_D of ≈0.5 μM for EGF binding to monomeric EGFR-ED. However, they report a $k_{off} \approx 0.05$ s^{-1} and a $k_{on} \approx 1 \times 10^5$ M^{-1} s^{-1}. In this work, they employ the technique of surface plasmon resonance for biomolecular interaction analysis (BIAcore). This method follows in real time the association and dissociation of receptor−ligand complexes as an injected solution of EGFR-ED passes over immobilized EGF and the difference in refractive index is measured. Using experimental methods similar to those of the Schlessinger lab, similar kinetic parameters and equilibrium constants were found when TGF-α was the immobilized ligand (M. O'Connor-McCourt, unpublished results). Nonetheless, these k_{off} values are 10^3-10^4 less than the k_{off} determined from our NMR results and their k_{on} values are correspondingly 10^3-10^4 less than a diffusion controlled k_{on}. A k_{off} of 0.05 s^{-1} would place the chemical exchange rate in the slow

chemical exchange regime on the NMR time scale. Our data clearly show that this is not the case. It is possible that the two techniques give different results; specifically immobilization of the receptor or ligand may influence the kinetics, or the rate constants may be too rapid to be accurately measured by the BIAcore technique. Further, their experimental conditions were slightly different and the ligand was different. Nonetheless, these details/differences are not likely to account for the several orders of magnitude difference between these two methods. Further, the very slow k_{on} measured by the Schlessinger lab (Zhou et al., 1993) implies that a simple binding mechanism such as eq 4 is not sufficient to describe the results (Baldo et al., 1975; Gutfreund, 1971, 1972; Hammes et al., 1970). Zhou et al. (1993) postulate a more complex mechanism involving receptor dimerization. Another proposal that can accommodate both the NMR and plasmon resonance results is a binding mechanism such as

$$\text{TGF}\alpha + \text{EGFR-ED} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{TGF}\alpha\cdot\text{EGFR-ED} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (\text{TGF}\alpha\cdot\text{EGFR-ED})^* \quad (7)$$

which has been found very commonly in situations where the measured k_{on} is so much less than the diffusion-controlled rate (Baldo et al., 1975; Gutfreund, 1971, 1972; Hammes et al., 1970). In this mechanism, the formation of a initial rapid recognition complex is followed by a conformational change to form a second complex. We can then postulate that the complex in rapid exchange observed in the NMR is TGFα·EGFR-ED and the complex dissociating slowly in the plasmon resonance experiments is (TGFα·EGFR-ED)*. In this mechanism the Zhou et al. (1993) value of k_{off} is associated with k_{-2}, and then k_{on} becomes k_1/K_{D1}. Extrapolating further, one might postulate that the TGFα·EGFR-ED to (TGFα·EGFR-ED)* conformational change is important in triggering receptor dimerization.

The plasmon resonance techniques also indicate that the exchange rate constants are pH dependent with k_{off} decreasing and k_{on} remaining constant at higher pH (M. O'Connor-McCourt, unpublished data). In terms of the proposed mechanism (eq 7) this would lead to a shift from TGFα·EGFR-ED to (TGFα·EGFR-ED)* and a reduction in the linebroadening from the TGFα + EGFR-ED $\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$ TGFα·EGFR-ED exchange since the high molecular weight complex (≈90 kDa) in slow exchange would be too broad for observation by NMR. To check this, 1-D ^{1}H NMR spectra of TGF-α in the presence of EGFR-ED were examined as a function of pH. In Figure 7, the 1-D NMR spectra of TGF-α in the presence of EGFR-ED at a mole ratio of 10:1 is shown for pH 6.0 (bottom) and pH 8.0 (top), respectively. The line broadening of the TGF-α resonances seen previously [in Figure 3 (pH 6.0)] in the presence of EGFR-ED is seen to be dramatically reduced at pH 8.0 so that the TGF-α resonances return to narrow lines. This can be seen most clearly for A50. Resonances in other parts of the spectra also become narrower; however, the exact chemical shifts of the methyl resonances are somewhat different due to the change in pH. The narrow line widths seen in Figure 7 are consistent with the strong pH dependence of the k_{off} rate.

If the mechanism shown in eq 7 is valid, then the bound line width (R_{2p_max}) shown in Figure 5C reflect the line broadening of resonances in the TGFα·EGFR-ED complex.

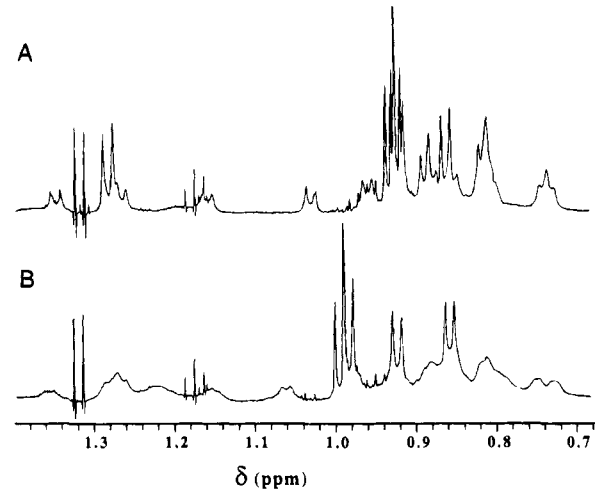

FIGURE 7: Comparison of the 1-D ^{1}H NMR spectrum of TGF-α in the presence of EGFR-ED (10:1) at pH 6 and 8. The top spectra (A) is recorded at pH 8.0 and the bottom (B) at pH 6.0. Each spectrum represents 512 scans with a digital resolution of 0.25 Hz/pt (32K data points collected). Processing was accomplished using resolution enhancement with window functions of −1.60 = lb and 0.60 = gf. Spectra were recorded at 298 K and at 600 MHz. The TGF-α concentration 1.2 mM and EGFR-ED at 0.12 mM. Peaks at 1.18 ppm (triplet) and 1.32 ppm (doublet) are small molecule impurities.

The relaxation measurements reflect the overall motion of the protein, the differential motion of the backbone and side chain residues, and the internal motion of the methyl groups. For free TGF-α the differential mobility was greatest for the N- and C-termini which were found to be much more mobile than the rest of the molecule. Upon binding the N terminus remains quite mobile relative to the C-terminus and the interior core methyl groups as evidenced by the line broadening (Figure 3) and relaxation measurements (Figure 5). When comparing C-terminus to the residues of the interior core, the C-terminus undergoes a severe restriction in its mobility upon binding to receptor. However, when comparing the R_{2p_max} values in the presence of receptor (Figure 5C), these values are similar for methyl groups in the C-terminus and the interior core (e.g., L48/49 and L24). This result implies that the motion is similar for both regions in the presence of receptor.

To discuss these results, we need to estimate the line-broadening of the fully immobile ligand bound to the receptor. From eq 3, we calculate $R_{2p\_max} = 225\ s^{-1}$ in the presence of no internal motion other than methyl rotation which will persist for bound TGF-α, estimating τ_c from the Stokes−Einstein equation (Brauer & Sykes, 1987) as 60×10^{-9} s. We can see in Figure 5C that the calculated R_{2p_max} is much greater than N-terminal V1 and V2 residues, slightly more than the A + B loop residues T13, T20, L24, V25, and A31 and smaller than the C loop residues. One must acknowledge the large experimental error in the slopes of Figure 5A because some resonances overlap, and overlap increases with added receptor and concomitant line broadening, and therefore the R_{2p_max} for some resonances (T20, V39, and A50) were determined from only a few points. Alternatively, the extra line broadening for C loop residues may be the result of exchange line broadening which occurs for resonances which are chemical shifted when bound (Sykes & Scott, 1972). Another important potential source of line broadening for the bound TGF-α methyl resonances comes

via relaxation with receptor nuclei. Thus, methyl resonances of the C loop in contact with receptor may have additional relaxation pathways available that other methyl resonances do not. Nonetheless, the line broadening is near appropriate values and greatest in regions shown by other data to be important for binding, i.e., residues defined by site-directed mutagenesis to be critical (Defeo-Jones et al., 1988, 1989; Lazar et al., 1988, 1989; Engler et al., 1988, 1990, 1991; Matsunanmi et al., 1991). The most obvious contrast is the lack of immobility of N-terminal residues.

The finding that the N-terminus is very mobile in the initial TGFα·EGFR-ED complex may be taken as a indication that no contact is made with this region with the receptor. However, this does not prove that this region makes no important contacts in the (TGFα·EGFR-ED)* state either with the monomeric receptor or upon subsequent formation of an activated dimer. The N-termini or C-termini of a variety of cytokines have been recently shown to be important in the activity of these cytokines with their receptors (Clark-Lewis et al., 1991; Ember et al., 1991). Specifically, for IL-8 analogs with deletion of critical N-terminal residues serve as good antagonists. Much less is known about the mode of activation of these systems and no direct physical studies of bound-ligand flexibility have been done. Thus, the present hypotheses might suggest that N-terminal or C-terminal deleted TGF-α analogs would be good antagonists and not lead to further activation. Future studies are in progress to address these issues of fundamental importance.

ACKNOWLEDGMENT

We thank Drs. Krishna Rajarathnam, Key-sun Kim, and Jian-jun Wang for their critical reading of the manuscript. We thank Paul Semchuk for his expert technical assistance in mass spectrometry of TGF-α. We are grateful to Perry d'Obrennan for his help in making figures. Finally, we thank Susan Smith and Colleen Iwanicka for their clerical assistance in making equations and tables.

REFERENCES

Anglister, J., & Naide, F. (1991) *Methods Enzymol. 203*, 228−241.

Baldo, J. H., Halford, S. E., Patt, S. L., & Sykes, B. D. (1975) *Biochemistry 14*, 1893−1899.

Baron, M., Norman, D. G., Harvey, T. S., Hanford, P. A., Mayhew, M., Tse, A. G., Brownlee, G. G., & Campbell, I. D. (1992) *Protein Sci. 1*, 81−90.

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355−360.

Bodenhausen, G., Kolger, H., & Ernst, R. R. (1984) *J. Magn. Reson. 58*, 370−388.

Brauer, M., & Sykes, B. D. (1987) in *Phosphorus NMR in Biology* (Burt, C. T., Ed.) pp 153−184, CRC Press, Boca Raton, FL.

Brown, P. M., Debanne, M. T., Grothe, S., Bergsma, D., Caron, M., Kay, C. M., & O'Connor-McCourt, M. D. (1994) *Eur. J. Biochem.* (in press).

Brown, S. C., Weber, P. L., & Mueller, L. (1988) *J. Magn. Reson. 53*, 521−528.

Campbell, A. P., & Sykes, B. D. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 99−122.

Carpenter, G. (1987) *Annu. Rev. Biochem. 56*, 881−914.

Carpenter, G., & Cohen, S. (1990) *J. Biol. Chem. 265*, 7709−7712.

Cheng, J. W., Lepre, C. A., Chambers, S. P., Fulghum, J. R., Thomson, J. A., & Moore, J. M. (1993) *Biochemistry 32*, 9000−9010.

Cheng, J. W., Lepre, C. A., & Moore, J. M. (1994) *Biochemistry 33*, 4093−4100.

Clark-Lewis, I., Schumacher, C., Baggiolini, M., & Moser, B. (1991) *J. Biol. Chem. 266*, 23128−23134.

Cooke, R. M., Wilkinson, A. J., Baron, M., Pastore, A., Tappin, M. J., Campbell, I. D., Gregory, H., & Sheard, B. (1987) *Nature 327*, 339−341.

Darlak, K., Franklin, G., Woost, P., Soonenfeld, E., Twardzik, D., Spatola, A., & Schultz, G. (1988) *J. Cell Biochem. 36*, 341−352.

Debanne, M. T., Pacheco-Oliver, M. C., & O'Connor-McCourt, M. D. (1994) in *Baculovirus Expression Protocols* (Richardson, C., Ed.) Humana Press, Totona, NJ.

Defeo-Jones, D., Tai, J. Y., Wegrzyn, R. J., Vuocolo, G. A., Baker, A. E., Payne, L. S., Garsky, V. M., Oliff, A., & Rieman, M. W. (1988) *Mol. Cell. Biol. 8*, 2999−3007.

Defeo-Jones, D., Tai, J. Y., Vuocolo, G. A., Wegrzyn, R. J., Scholfield, T. L., Oliff, A., & Rieman, M. W. (1989) *Mol. Cell. Biol. 9*, 4083−4086.

Derynck, R., Goeddel, D. V., Ullrich, A., Gutterman, J. U., Williams, R. D., Bringman, T. S., & Berger, W. H. (1987) *Cancer Res. 47*, 707−712.

deVos, A. M., Ultsch, M., & Kossiakoff, A. A. (1992) *Science 255*, 306−312.

Dougall, W. C., Qian, X., & Green, I. (1993) *J. Cell Biochem. 53*, 61−73.

Dwek, R. A. (1973) *Nuclear Magnetic Resonance (N.M.R.) in Biochemistry: Application to Enzyme Systems*, Claredon Press, Oxford.

Ember, J. A., Johnansen, N. L., & Hugli, T. E. (1992) *Biochemistry 30*, 3603−3612.

Engler, D. A., Matsunami, R. K., Campion, S. R., Stringer, C. D., Stevens, A., & Niyogi, S. K. (1988) *J. Biol. Chem. 263*, 12384−12390.

Engler, D. A., Montelione, G. T., & Niyogi, S. K. (1990) *FEBS Lett. 271*, 47−50.

Engler, D. A., Hauser, M. R., Cook, J. S., & Niyogi, S. K. (1991) *Mol. Cell. Biol. 11*, 2425−2431.

Eppstein, D. A., Marsh, Y. V., Schryver, B. B., & Bertics, P. J. (1989) *J. Cell. Physiol. 141*, 420−430.

Fesik, S. W., Gampe, R. T., Eaton, H. L., Gemmecker, G., Olejniczak, E. T., Neri, P., Holzman, T. F., Egan, D. A., Edalji, R., Simmer, R., Helfrich, R., Hochlowski, J., & Jackson, M. (1991) *Biochemistry 30*, 6574−6583.

Goldman, M. (1988) *Quantum Description of High-Resolution NMR in Liquids, International Series of Monographs on Chemistry 15* (Rowlinson, J. S., Ed.) pp 243−248, Clarendon Press, Oxford.

Grasberger, B. L., Gronenborn, A. M., & Clore, G. M. (1993) *J. Mol. Biol. 230*, 364−372.

Griesinger, C., Otting, G., Wüthrich, K., & Ernst, R. R. (1988) *J. Am. Chem. Soc. 110*, 7870−7872.

Grimaux, M., Laine-Bidron, C., & Magdelenat, H. (1989) *Tumor Biol. 10*, 215−224.

Gutfreund, H. (1971) *Annu. Rev. Biochem. 40*, 315−344.

Gutfreund, H. (1972) *Enzymes: Physical Principles*, Wiley-Interscience, London.

Harvey, T. S., Wilkinson, A. J., Tappin, M. J., Cooke, R. M., & Campbell, I. D. (1991) *Eur. J. Biochem. 198*, 555−562.

Hammes, G. G., & Schimmel, P. R. (1970) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. 2, pp 67−114, Academic Press, New York.

Heath, W. F., & Merrifield, R. B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6367−6371.

Hoeprich, P. D., Langton, B. C., Zhang, J., & Tam, J. P. (1989) *J. Biol. Chem. 264*, 19086−19091.
Hommel, U., Harvey, T. S., Driscoll, P. C., & Campbell, I. D. (1992) *J. Mol. Biol. 227*, 271−282.
Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., & Bax, A. (1992) *Science 256*, 632−638.
Jardetzky, O., & Roberts, G. C. K. (1981) *NMR in Molecular Biology*, Academic Press, New York.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546−4553.
Katsuura, M., & Tanaka, S. J. (1989) *J. Biochem. (Toyko) 106*, 87−92.
Kline, T. P., Brown, F. K., Brown, S. C., Jeffs, P. W., Kopple, K. D., & Mueller, L. (1990) *Biochemistry 29*, 7805−7813.
Kohda, D., Shimada, I., Miyake, T., Fuwa, T., & Inagaki, F. (1989) *Biochemistry 28*, 953−958.
Korc, M., & Finman, J. E. (1989) *J. Biol. Chem. 264*, 14990−14999.
Kumar, A., Ernst, R. R., & Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun. 95*, 1−6.
Lax, I., Fischer, R., Ng, C., Segre, J., Ullrich, A., Givol, D., & Schlessinger, J. (1991) *Cell Regul. 2*, 337−345.
Lazar, E., Watanabe, S., Dalton, S., & Sporn, M. B. (1988) *Mol. Cell. Biol. 8*, 1247−1252.
Lazar, E., Vicenzi, E., Van Oberghen-Schilling, E., Wolff, B., Dalton, S., Watanabe, S., & Sporn, M. B. (1989) *Mol. Cell. Biol. 9*, 860−864.
Luetteke, N. C., Qiu, T. H., Peiffer, R. L., Oliver, P., Smithies, O., & Lee, D. C. (1993) *Cell 73*, 263−278.
Mann, G. B., Fowler, K. J., Gabriel, A., Nice, E. C., Williams, R. L., & Dunn, A. R. (1993) *Cell 73*, 249−261.
Marshall, A. G., Schmidt, P. G., & Sykes, B. D. (1972) *Biochemistry 11*, 3875−3879.
Martin, P., Hopkinson-Wooley, J., & McCluskey, J. (1992) *Prog. Growth Factors Res. 4*, 25−44.
Matsunami, R. K., Yette, M. L., Stevens, A., & Niyogi, S. K. (1991) *J. Cell. Biochem. 46*, 242−249.
Meiboom, S., & Gill, D. (1958) *Rev. Sci. Instrum. 29*, 688−691.
Miller, J. H. (1972) in *Experiments in Molecular Genetics*, p 431, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Montelione, G. T., Wüthrich, K., Burgess, A. W., Nice, E. C., Wagner, G., Gibson, K. D., & Scheraga, H. A. (1992) *Biochemistry 31*, 236−249.
Moy, F. J., Li, Y.-C., Ravenbuehler, P., Winkler, M. E., Scheraga, H. A., & Montelione, G. T. (1993) *Biochemistry 32*, 7334−7353.
Padmanabhan, K., Padmanabhan, K. P., Tulinsky, A., Park, C. H., Bode, W., Huber, R., Blankenship, D. T., Cardin, A. D., & Kisiel, W. (1993) *J. Mol. Biol. 232*, 947−966.
Palmer, A. G., Hochstrasser, R. A., Millar, D. P., Rance, M., & Wright, P. E. (1993) *J. Am. Chem. Soc. 115*, 6333−6345.
Piatini, U., Sorensen, O. W., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 6800−6801.
Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479−485.
Richardson, J. S., Richardson, D. C., Tweedy, N. B., Gernert, K. M., Quinn, T. P., Hecht, M. H., Erickson, B. W., Yan, Y., McClain, R. D., Donlan, M. E., & Surles, M. C. (1992) *Biophys. J. 63*, 1186−1209.
Richter, A., Conlan, J. W., Ward, M. E., Chamberlain, S. G., Alexander, P., Richards, N. G. J., & Davies, D. E. (1992) *Biochemistry 31*, 9546−9554.
Rousseau, D. L., Guyer, C. A., Beth, A. H., Papayannopoulos, I. A., Wang, B., Wu, R., Mroczkowski, B., & Staros, J. V. (1993) *Biochemistry 32*, 7893−7903.
Spear, K. L., & Sliwkowski, M. X. (1991) in *Techniques in Protein Chemistry II* (Villafranca, J. J., Ed.) pp 233−240, Academic Press, New York.
Staros, J. V., Fanger, B. O., Faulkner, L. A., Palaszewski, P. P., & Russo, M. W. (1989) in *Receptor Phosphorylation* (Moudgil, V. K., Ed.) pp 227−242, CRC Press, Boca Raton, FL.
States, D. J., Haberkorn, R. A., & Rueben, D. J. (1982) *J. Magn. Reson. 48*, 286−292.
Sykes, B. D. (1993) *Curr. Opin. Biotechnol. 4*, 392−396.
Sykes, B. D., & Scott, M. D. (1972) *Annu. Rev. Biophys. Bioeng. 1*, 27−50.
Sykes, B. D., Hull, W. E., & Snyder, G. H. (1978) *Biophys. J. 21*, 137−146.
Tam, J. P., Lin, Y.-Z., Liu, W., Wang, D.-X., Ke, X.-H., & Zhang, J.-W. (1991) *Int. J. Pept. Protein Res. 38*, 204−211.
ten Dijke, P., & Iwata, K. K. (1989) *Bio/Technology 1*, 793−798.
Vold, R. L., Waugh, J. S., Klein, M. P., & Phelps, D. E. (1968) *J. Chem. Phys. 48*, 3831−3832.
Werbelow, L. G., & Marshall, A. G. (1974) *Mol. Phys. 28*, 113−129.
Werbelow, L. G., & Grant, D. M. (1977) *Adv. Magn. Reson. 9*, 189−299.
Zhou, M., Felder, S., Rubinstein, M., Hurwitz, D. R., Ullrich, A., Lax, I., & Schlessinger, J. (1993) *Biochemistry 32*, 8193−8198.