NMR Study of the Transforming Growth Factor-α (TGF-α)-Epidermal Growth Factor Receptor Complex

VISUALIZATION OF HUMAN TGF-α BINDING DETERMINANTS THROUGH NUCLEAR OVERHAUSER ENHANCEMENT ANALYSIS*

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The study of human transforming growth factor-α (TGF-α) in complex with the epidermal growth factor (EGF) receptor extracellular domain has been undertaken in order to generate information on the interactions of these molecules. Analysis of 1H NMR transferred nuclear Overhauser enhancement data for titration of the ligand with the receptor has yielded specific data on the residues of the growth factor involved in contact with the larger protein. Significant increases and decreases in nuclear Overhauser enhancement cross-peak intensity occur upon complexation, and interpretation of these changes indicates that residues of the A- and C-loops of TGF-α form the major binding interface, while the B-loop provides a structural scaffold for this site. These results corroborate the conclusions from NMR relaxation studies (Hoyt, D. W., Harkins, R. N., Debanne, M. T., O’Connor-McCourt, M., and Sykes, B. D. (1994) Biochemistry 33, 15283–15292), which suggest that the C-terminal residues of the polypeptide are immobilized upon receptor binding, while the N terminus of the molecule retains considerable flexibility, and are consistent with structure-function studies of the TGF-α/EGF system indicating a multidomain binding model. These results give a visualization, for the first time, of native TGF-α in complex with the EGF receptor and generate a picture of the ligand-binding site based upon the intact molecule. This will undoubtedly be of utility in the structure-based design of TGF-α/EGF agonists and/or antagonists.

Human TGF-α is a 50-amino acid polypeptide with 40% sequence homology to epidermal growth factor (1, 2). In addition, the structural similarity of the two molecules results in their ability to compete for binding to the EGF receptor (3–5). Complexation of TGF-α with this receptor is believed to mediate a variety of biological effects, including embryonic development of certain tissues and wound healing (6, 7); however, the major sphere of interest of this protein lies in its role in the transformation and maintenance of various malignant tumors (8, 9).

The structural features of the homologous growth factors that contain three disulfides and hence three loops (A, B, and C) have been determined by NMR (10–13) and include a triple-stranded anti-parallel β-sheet comprising the N-terminal region, a smaller anti-parallel double hairpin in the C terminus of the molecule, and a helical segment in the A-loop in some structures. Previous studies undertaken to elucidate the structurally important residues of TGF-α (and EGF) required for complexation have implicated residues including Phe-15, Tyr-38, Arg-42, and Leu-48 (14–18). The consensus of a variety of structural studies including the use of synthetic peptide fragments (19–21), recombinant chimeric proteins (22, 23), and anti-TGF-α and anti-EGF antibodies (24, 25) is that receptor binding occurs with multiple domains of TGF-α, although conflicting results have been obtained concerning the involvement of the A-, B-, and C-loops. The multidomain binding model is consistent with the observation that, at present, it is not possible to reduce the size of the growth factor without significantly compromising its affinity for the EGF receptor. This was illustrated by deletion studies where the N-terminal residues outside the A-loop were truncated. This mutant had 3% of the binding affinity of the intact protein (20). Further data from receptor-bound TGF-α may lead to the structure-based design of reductant molecules through the precise identification of ligand binding determinants.

Hoyt et al. (26) have recently demonstrated, through a study of 1H NMR transverse and longitudinal relaxation rates for the methyl resonances of TGF-α in the free state and in association with the EGFR-ED, that the C-terminal residues undergo a dramatic decrease in flexibility upon binding, while the N terminus maintains a degree of mobility similar in both bound and free forms of the ligand. The mid-portions of the molecule underwent a moderate decrease in flexibility relative to the uncomplexed polypeptide. The conclusions of this work are consistent with the previous studies, which suggest that the C-terminal residues are responsible for receptor binding, while the B-loop provides a structural scaffold for the primary site of interaction.

This study presents new insight into the components of the TGF-α structure that are requisite for complex formation from...
the analysis of changes in the two-dimensional $^1$H NMR NOESY spectra of the ligand upon titration with the EGFR-ED (molecular mass of 85 kDa). NMR is the method of choice in the elucidation of the ligand contact sites with the receptor since it is the only technique that can give detailed information on these molecules in solution. The results suggest the involvement of residues in the A- and C-loops and C-terminal tail in the primary site of interaction of the growth factor with its receptor.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of TGF-α—**TGF-α and the EGFR-ED were prepared and purified according to previous methods (26).

**NMR Sample Preparation—**To a lyophilized sample of TGF-α was added 460 µl of buffer containing 50 mM potassium phosphate, 10 mM potassium chloride, 1 mM EDTA, 0.5 mM sodium azide, 0.15 mM sodium carbonate 2.2-dimethyl-2-silapentane-5-sulfonate (internal standard), and 99.9% D$_2$O or 90/10% (v/v) H$_2$O/D$_2$O. The solution was adjusted to pH 6.0 by the addition of small aliquots of 0.5 N NaOD or 0.5 N HCl, bringing the final volume to 500 µl. 230-µl aliquots of a stock EGFR-ED solution that had previously been dialyzed against the above buffer were then added successively to the TGF-α NMR sample to give concentrations of 0, 2.4, 4.9, and 6.5% EGFR-ED.

**$^1$H NMR Spectroscopy—**$^1$H NMR spectra for TGF-α free in solution and in the presence of various amounts of EGFR-ED were acquired at 599.9 MHz using a Varian Unity 600 spectrometer. These included one-, two-, and three-dimensional experiments collected at 298 K, referenced relative to an internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate standard and utilizing presaturation to attain suppression of the water resonance. The hypercomplex method (27) was used for acquisition of two-dimensional NOESY spectra (28–30), which incorporated 40, 64, 96, and 120 transients (for 0, 2.4, 4.9, and 6.5% EGFR-ED titration points, respectively) for each of 256 increments. NOESY spectra were recorded using 50-, 100-, and 150-ms mixing times at each receptor concentration. Each spectrum employed a spectral width of 8000 Hz and 2048 data points. The Fourier transformation of the spectra utilized shifted sinebell and zero filling to 4096 points in both dimensions. The three-dimensional $^{15}$N edited NOESY (HMOC-NOESY) spectrum was acquired using a mixing time of 150 ms with the proton carrier frequency set to 4.73 ppm and a presaturation pulse to suppress the H$_2$O peak. The experiment was collected with sweep widths of 8000 Hz and 2048 data points. The Fourier transformation of the spectra utilized shifted sinebell and zero filling to 4096 points in both dimensions. The three-dimensional $^{15}$N edited NOESY (HMOC-NOESY) spectrum was acquired using a mixing time of 150 ms with the proton carrier frequency set to 4.73 ppm and a presaturation pulse to suppress the H$_2$O peak. The experiment was collected with sweep widths of 8000 Hz and 2048 data points. The Fourier transformation of the spectra utilized shifted sinebell and zero filling to 4096 points in both dimensions.

**RESULTS**

Two-dimensional $^1$H NMR NOESY spectra of TGF-α free in solution and in complex with the EGFR receptor at different [TGF-α]/[EGFR-ED] ratios were performed in order to generate structural information on the bound conformation of TGF-α, which could be used to determine the specific regions and residues of the ligand involved in binding. Using the transferred NOE methodology, a ligand in fast exchange with a receptor molecule is studied in excess (usually 10:1 to 20:1) over the receptor. During the course of the NOESY experiment, the bound ligand magnetization is transferred to the excess free ligand through exchange and thus, the NMR information characterizing the bound structure is observed via the sharp resonances of the free ligand. At pH 6.0, TGF-α is in fast exchange with the EGFR-ED and has an off-rate of ≥ 300 s$^{-1}$ (26), and therefore, this system has conditions amenable to study by TR-NOESY. The complete assignment of proton NMR resonances of TGF-α at pH 6.0 has been previously reported (26) and was utilized in order to assign the cross-peaks for a series of TR-NOESY spectra of TGF-α free and in the presence of 0, 2.4, 4.9, and 6.5% EGFR-ED. From the 150-ms NOESY data, a total of 544 cross-peaks in the free ligand, 419 were assigned unambiguously. Of the 419 unambiguous cross-peaks, 404 were assigned from the two-dimensional NOESY spectrum and 15 were additionally assigned from the three-dimensional HMQC-NOESY spectrum as the latter were able to be resolved in the $^{15}$N dimension from the three-dimensional spectrum.

After assignment of the TR-NOESY cross-peaks for the EGFR-ED titration series, it became apparent that very few new peaks were observed in the spectra. Due to the absence of new cross-peaks or observable chemical shift differences between the free and bound ligand, it was concluded that no major structural rearrangement of TGF-α occurs upon complexation. However, a systematic study of the spectra obtained for each point of the TGF-α/EGFR-ED titration revealed specific information on the sites of interaction of the growth factor. This was achieved by ranking the cross-peaks by the slope of the NOE intensity changes during the receptor titration series. For each spectrum in the series, the NOE volume is first divided by the average intensity in order to more accurately assess the changes between spectra. For the addition of 2.4% EGFR-ED, 524 NOEs were observed in the 150-ms NOESY spectrum, indicating that 20 peaks had disappeared. For the subsequent aliquot, bringing the receptor concentration to 4.8%, the corresponding spectrum contained 476 NOEs, and thus, 48 additional NOEs had disappeared. For the final aliquot (6.5% EGFR-ED), 434 NOEs were observed, and thus, a further 42 were absent in this spectrum. For the peaks that remained over the course of the titration, 186 increased in intensity, and 247 decreased in intensity. Fig. 1 illustrates the amide and aromatic connectivities of the TR-NOESY spectra of TGF-α, free (panel A) and in the presence of 6.5% EGFR-ED (panel B). It can be seen from these spectra that the majority of the cross-peaks decrease in intensity in the bound compared with the free ligand.

With the intention of elucidating the receptor contact sites, the NOEs that disappeared from 150-ms NOESY spectra over the course of the titration were examined. Cross-peak intensity in TR-NOESY spectra is determined by a large number of factors, including relaxation in the free and bound ligand, exchange rate, fraction bound, and NOESY mixing time. Relaxation in the free ligand is determined by internuclear distance and the rotational correlation times of the free ligand, while relaxation in the bound ligand is influenced by intranuclear distance, the rotational correlation time of the complex, and additional relaxation pathways involving contact between protons in the ligand and in the target protein. In general, when the ligand is a small flexible peptide, the intrinsic NOEs in the absence of protein are small because of the short correlation time and concomitant long cross-relaxation rates. In the complex with the target protein, the ligand assumes the correlation time of the target protein and extends the spin-diffusion limit ($\omega_0\tau_c$, $> 1$) where the NOEs are large. Under these circumstances, the TR-NOESY intensities generally increase as the fraction of ligand bound increases (for small fraction bound) at constant NOESY mixing time or increase with increasing NOESY mixing time (for short $\tau_m$) at constant fraction bound (33, 34). The ranges over which these limits are observed become more stringent as the size of the target protein increases. However, the situation is more complicated when the ligand is also a protein such as TGF-α for which...
significant NOESY cross-peaks already exist and where differential mobility exists in both the free and bound states, as is also the case with TGF-\(\alpha\) (26). Thus, one can expect TR-NOESY cross-peaks to both increase and decrease. A priori, one would expect shorter bound internuclear distance and increased correlation time to increase TR-NOESY cross-peak intensity in the limit of small fraction bound and short NOESY mixing times, but the effect can be attenuated by rapid cross-relaxation. Longer distances and additional relaxation pathways involving ligand protons would lead to a decrease in cross-peak intensity at small fraction bound and short mixing times.

To quantitate the effects of the competing influences in TR-NOESY cross-peak intensity, we have simulated spectra with estimates of the free and bound correlation times (3 and 40 ns, respectively) and exchange rates (500 s\(^{-1}\)) using a program developed for Mathematica and kindly provided by R. London (35) and subsequently modified by one of us (B. D. S.). The simulations indicate that the change in the TR-NOESY intensity is approximately linear in fraction bound for the values of fraction bound and mixing time used in this study, as is observed experimentally. Increases in intensity expected from the increased bound rotational correlation time are slight at the fraction bound used. The most striking effects come when additional relaxation pathways are considered that lead to a decrease in cross-peak intensity at small fraction bound and short mixing times.

Therefore, it was decided that the most unambiguous TR-NOESY cross-peaks to focus on were those that decrease, possibly caused by increased internuclear distance, but most likely as a result of increased relaxation caused by spin-diffusion contact with protons on the target protein. Although there is an overall decrease in NOE intensity throughout the TGF-\(\alpha\) molecule, corresponding increases in intensity of a number of cross-peaks are observed (one-third of the total number of NOEs are more intense in the TGF-\(\alpha\)-EGFR-ED complex).

Since there are a number of pathways by which the intensity increases can occur, their interpretation is not straightforward; however, since it is probable that magnetization bleed-off occurs from ligand contact sites, it can be surmised that the atoms involved in NOEs that increase are those that either are non-surface protons or are not in contact with the EGF receptor, which typically experience the largest perturbations upon binding.

In addition to ligand magnetization loss to receptor protons, exchange broadening may play a significant role in the decreased NOE intensities. The interpretation of absent NOEs in the receptor-bound ligand is not affected in this case since these effects would be most pronounced for the residues and atoms in contact with the receptor.

For the case of the complexation of TGF-\(\alpha\) with the EGF receptor, in which the changes in the NOE intensity from the series of NOESY spectra were examined, the most striking changes were in the number of NOEs that disappeared over the course of the receptor titration. Of a total of 544 NOEs in the uncomplexed form of TGF-\(\alpha\), 110 NOEs were no longer present in the bound polypeptide (i.e. the final receptor titration point). As discussed, the disappearance of these peaks most probably occurs as a result of magnetization bleed-off from protons of the ligand that are in direct contact with the receptor.

From the changes in the NOE intensity upon receptor addition, an understanding of the molecular nature of the interactions of the TGF-\(\alpha\)-EGFR-ED complex can thus be deduced. As mentioned, of particular significance are the NOEs that are absent from the NOESY spectra of bound TGF-\(\alpha\). Fig. 2 illustrates a Connolly surface representation of the TGF-\(\alpha\) structure and shows the atoms involved in NOEs that disappear in the NOESY spectrum of the TGF-\(\alpha\)-6.5% EGFR-ED complex.

From Fig. 2, the observation can be made that almost the whole surface of the polypeptide has atoms that lose cross-peaks, with
Interestingly, these residues are localized on the TGF-α indicating that they are not immobilized by the receptor. Residues 1–4, 10, and 25–27 have no NOEs that disappear, the changes that are concomitant with receptor binding. First, Examination of Fig. 3 shows the total number of NOEs per residue and the percentage of NOEs that were absent upon complexation versus the number of NOEs per residue in the free ligand. Fig. 3 (A and B) shows the total number of NOEs per residue and the percentage of absent NOEs in the receptor-bound ligand, respectively. Examination of Fig. 3B reveals some striking information on the changes that are concomitant with receptor binding. First, residues 1–4, 10, and 25–27 have no NOEs that disappear, indicating that they are not immobilized by the receptor. Interestingly, these residues are localized on the TGF-α structure at the N-terminal subdomain embodying part of the triple-stranded β-sheet. The other noticeable features of the graph in Fig. 3B are the residues for which between 40 and 60% of the free ligand NOEs are absent. These include His-12, Thr-13, Phe-15, Phe-17, Ala-31, Val-39, Gly-40, His-45, Leu-48, and comprise segments of the A- and C-loops and the C-terminal tail of TGF-α. Also significant are those amino acids that lose between 20 and 40% of their cross-peaks upon complexation. If these are considered, almost all of the A-loop residues (from Ser-11 to Leu-24) are affected.

When the results shown in Fig. 3B are grouped into classes by percentage and visualized on the TGF-α structure, further insight into the ligand/receptor interactions is facilitated. Fig. 4 illustrates the NOE changes by color on the surface of TGF-α and on the secondary structure of the growth factor. The immediate observation from Fig. 4 is the localization of the different colored residues. The blue and purple colors (representing the least changes in terms of absent NOEs) are clustered, for the most part, in the N-terminal subdomain of the ligand. The white colors (indicating 20–30% of NOEs that disappear) are more disperse throughout the molecule; however, the green residues (30–40% NOEs absent) are grouped on one face, which comprises the central region of TGF-α on the B- and C-loops. The residues involved in the highest percentage of absent NOEs in the receptor-bound state (shown in red) are clustered in two groups, the major of which also constitutes one face of TGF-α. This face consists of residues in the A- and C-loops and the C-terminal tail of the ligand. This face also includes Glu-44, which loses 30–40% of NOEs upon binding, and is contiguous with His-12, Phe-15, Phe-17, Val-39, Gly-40, and His-45 (which lose 40–60% of NOEs) on the surface of the molecule. Leucines 48 and 49 on the C-terminal tail of TGF-α both lose 50% of their NOEs, indicating that these residues play a significant role in the binding interface of the ligand-receptor complex.

Examination of the NOEs in terms of the intramolecular, intermolecular, and long-range (i,i+2 or further) NOEs that disappear indicates that they have similar distribution as occurs in the free ligand, i.e. in the NOESY spectrum of the bound ligand, 29, 36, and 39% of the intramolecular, intermolecular, and long-range NOEs, while in free TGF-α, the values are 35, 34, and 31%, respectively. This suggests that the structure is not significantly altered upon binding and that one class of NOE is not greatly more affected than another. If the case were considered where a significantly higher percentage (relative to the free ligand) of intermolecular rather than intramolecular NOEs disappeared upon binding, then this would suggest that structural perturbations rather than complexation were responsible.

Further inspection of the NOE data provides information on receptor binding that augments that previously discussed. Fig. 5 shows the number of NOEs that disappear upon addition of the first receptor aliquot. Of particular note are the most intense NOEs that are absent in the receptor-bound form. These NOEs are suggestive of the residues that form the strongest interaction with the EGF receptor since they disappear at the lowest receptor concentration. The majority of these correlate with those residues identified in the previous interpretation (Fig. 4) and thus predicate the implied binding interface.

**DISCUSSION**

The NOE analysis method for elucidating the receptor contact sites of the ligand is discussed in light of the current
understanding of the molecular requirements for TGF-α complex formation with the EGF receptor. A plethora of studies have been undertaken to determine the components of the TGF-α/EGF system that are requisite for receptor binding and activation; however, these as a whole have failed to provide a consensus as to the essential residues and regions (19–25). It has been established that a correct native fold of both growth factors is critical for biological activity since the disruption of any single disulfide results in complete loss of binding (14). Deletion studies using synthetic peptides indicate that virtually no part of these polypeptides can be removed without a significant decrease in activity. Even removal of the flexible N-terminal tail in the case of TGF-α yields an analog with only 3% of the binding affinity of the native molecule (20).

Studies of EGF and TGF-α mutants in which single amino acids are replaced in a conservative or nonconservative fashion have, for the most part, produced conflicting results. Despite this, the critical role of certain residues in receptor binding and activation has emerged, including Phe-15, Tyr-38, Arg-42, and Leu-48 (14–18).

The results obtained by the NOE analysis method support a multidomain model for receptor binding as postulated (19–25) and shown in Fig. 4. The majority of the most important residues for EGF receptor complex formation as suggested by the residues that lose the highest percentage of NOEs are presented on a common face of TGF-α that comprises the A- and C-loops and consists of His-12, Phe-15, Phe-17, Val-39, Gly-40, and His-45, thus strongly implicating this face as a binding determinant for the ligand/receptor interaction. This postulation is corroborated by a previous study that demonstrated that antibodies specific for an epitope on the opposite face, consisting of the residues of the B-loop, were non-neutralizing in terms of receptor binding and thus proposed that the face of TGF-α including residues 12–20 and 34–43 was involved in binding (25). When the residues for which 30–40% of the NOEs were absent in the bound ligand were included in the face, a more extensive contiguous surface for receptor binding was apparent. This surface now includes two of the critical residues (Arg-42 and Phe-15) that lose 31 and 46% of their NOEs, respectively. Arg-42, which appears to be less important for binding based on the NOE criterion, may play a structural role in preserving the local conformation of Phe-15, which is a critical residue based upon the NOE data. Structural studies of the inactive R42K mutant exclude any gross conformational changes; however, do not rule out subtle effects that alter the microenvironment of the phenylalanine (17, 36).

A recent study on a chimeric growth factor consisting of the A- and C-loops of EGF and the B-loop of TGF-α concluded that,
since the hybrid protein exhibited enhanced binding to the receptor relative to EGF, the B-loop is an important determinant for receptor binding and mitogenic activity (22). This statement is undoubtedly true due to the multiplicity of binding domains; however, our results suggest that the B-loop plays more of a structural role in providing a molecular scaffold for presentation of the A- and C-loops to the receptor. In the case of the chimeric protein, the enhanced activity probably results from the increased stability of the A- and C-loop conformation effected by the B-loop. Another study from the same group reported mitogenic activity for a constrained B-loop analog of TGF-\(\alpha\) (37); however, it was very low relative to the native molecule and thus does not eliminate the role of A- and C-loop residues since the latter have been shown to contribute significantly to binding and activation of the receptor (14–18).

From Fig. 4, it is apparent that leucines 48 and 49 are immobilized in the receptor-bound ligand; however, it is also evident that these residues are not contiguous with the previously mentioned receptor interface. This observation implies that these residues provide a second interface that is integral for receptor complex formation. The impotency of the L48A analog of TGF-\(\alpha\) (18) corroborates the role of this face as a second anchor point to the EGF receptor.

Site-directed mutagenesis of Tyr-38 in TGF-\(\alpha\) and Tyr-37 in
EGF apparently gives conflicting results for the significance of this residue in the receptor interaction since it was shown to be nonessential in EGF (38) and essential in TGF-α (16). This conflict may be the consequence of a different mechanism or site of binding with the two ligands; however, our NOE data indicate that if mutation of this residue precludes binding of TGF-α, then it does so by altering the ligand conformation and thus probably does not contribute directly to the interaction.

If the NOEs that disappear upon addition of the first receptor aliquot are considered, then further insight into the receptor contacts is obtained. Of special note in the consideration of these NOEs are those of the greatest intensity that are absent in the spectrum of the first point of the titration. It can be envisaged that these NOEs belong to residues of TGF-α bound most tightly to the EGFR-ED. These residues include His-12, Phe-15, Phe-17, Phe-23, Val-39, His-45, and Leu-49 and, for the most part, predetermine the postulated binding faces and the contents of the analog studies.

It can be observed from Fig. 4 that the green residues (30–40% absent NOEs) are clustered on the view of TGF-α on the right. It is tempting to suggest that this face forms a binding interface for a second EGF receptor molecule and is of lower affinity than the face illustrated on the left Connolly surface representation in Fig. 4. Obviously, further experiments would be required to confirm this, although it has been proposed that the TGF-α/EGF/EGFR system is similar to that of human growth hormone, where one hormone binds two receptor molecules and some evidence for this mechanism has been discussed (39).

Hoyt et al. (26) have recently published a detailed study of methyl relaxation rates of TGF-α both free in solution and in complex with the receptor. Since these results and the current study were performed under the same conditions, congruity is to be expected between the two methods of analyzing the ligand/receptor interactions. Comparison of the transverse relaxation rates for all of the methyl-containing residues of TGF-α was used to delineate the relative mobilities of these residues within the TGF-α structure. Val-1 and Val-2 showed the highest mobility when receptor bound with virtually no enhancement of R₂ upon receptor binding. Since these two residues are flexible in both the bound and free forms, they are largely devoid of NOEs, and thus, the NOE comparison with the relaxation data cannot be made. Thr-13, Thr-20, Leu-24, Val-25, and Ala-31 all undergo intermediate loss of flexibility as determined from the R₂ values. This compares favorably with the NOE data (Fig. 6), which suggest, with the exception of Val-25, that these residues are in contact with the receptor. Val-25, which is part of the β-turn of the β-sheet in the B-loop, does not lose any NOEs upon receptor binding, thus suggesting that this residue is not involved in receptor binding. Its methyl relaxation, however, indicates that its mobility is somewhat restricted upon complexation. A possible explanation is that its mobility is restricted compared with the very flexible N-terminal tail, but it is still flexible enough that the NOEs of this residue, which may not be in direct contact with the receptor, thus do not disappear due to magnetization bleed-off. The residues of the C-loop and C-terminal tail show strong agreement between the relaxation and NOE data as these have both the largest relaxation enhancements and the largest percentage of NOEs that disappear upon complex formation.

The results of this study demonstrate that the NOE analysis method provides a model that explicates the current understanding of TGF-α/EGF interactions with the EGF receptor in terms of a multidomain model and provides significant information on the residues contributing to binding and activation.

**CONCLUSIONS**

Detailed analysis of the NOEs for free and bound species of TGF-α indicates that the majority of residues of the ligand that have the highest percentage of absent NOEs in the bound form embody one face of the molecule that is composed of the A- and C-loops. A second receptor anchor point is formed by the two C-terminal leucines. The NOE analysis results are consistent with relaxation studies that indicate restricted C-terminal mobility in bound TGF-α and with structure-function studies that suggest a multidomain ligand binding model. The elucidation of the ligand interaction sites is essential for the future development of TGF-α agonists and/or antagonists using structure-based drug design methodology.

**Acknowledgments**—We thank Dr. Krishna Rajarathnam for critical reading of this manuscript. We thank Paul Semchuk for expert technical assistance in mass spectrometry of TGF-α. In addition, we thank Susan Smith and Susan Henry for helpful clerical assistance.

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