Transforming growth factor β (TGF-β) form a group of multifunctional cytokines that account for a substantial portion of the intercellular signals governing cell fate (1). The TGF-β superfamily includes bone morphogenetic proteins (BMP), growth and differentiation factors, and activins/inhibins. Three mammalian TGF-β isoforms exhibit sequence homologies higher than 70% and are functionally closely related. They, however, show different biological activities in certain cell types or systems. Appropriate levels of TGF-β activity are essential to an organism’s well being (2). Lack of sufficient TGF-β can result in immunological and inflammatory disturbances, developmental abnormalities, deficient wound healing, and increased tumorigenesis. Conversely, excessive TGF-β activity leads to scarring, the development of fibrotic diseases in multiple organ systems, and immune suppression.

TGF-β is produced by virtually all cell types as an inactive precursor, which is then cleaved into a latent complex. Activation of latent TGF-β in vivo is caused by proteolytic cleavage of the latency-associated peptide (LAP), by enzymatic deglycosylation of LAP, by conformational changes of the latent complex following binding to thrombospondin, and by the acidification of the pericellular space (3, 4). To propagate signals across the cell membrane, the members of the TGF-β superfamily require two structurally related receptors (type I and type II), both having a short extracellular domain (ectodomain), a single membrane-spanning region, and an intracellular serine/threonine kinase domain (5). The members of the TGF-β superfamily are homodimers held together by a disulfide bond, and each monomer has binding sites for type I and type II receptors (6). During the signaling process TGF-β binds first to its type II receptor (TβR2) and then to type I receptor (TβR1). The ectodomain of TβR2 binds with higher affinity to TGF-β3 than to TGF-β1, whereas the recognition of TGF-β2 has to be supported by the presence of betaglycan (7). In the heteromeric complex of TGF-β with the receptors, the kinase domain of TβR2 phosphorylates TβR1, which in turn phosphorylates downstream intracellular signaling components (8).

The molecular basis for the diverse biological activities of TGF-β is not well understood. X-ray crystallographic (9, 10) and NMR (11) analyses have revealed identical folds of the TGF-β isoforms. However, recent studies have shown important structural differences of TGF-β3 in solution with respect to its crystal structure and the structures of other TGF-β isoforms. In view of the therapeutic potential of TGF-β, we present here a dynamic study of TGF-β3 aimed at explaining its behavior in aqueous solution and characterizing determinants involved in the interaction with receptors.

EXPERIMENTAL PROCEDURES

15N Relaxation Measurements—Biologically active, recombinant human TGF-β3 was prepared at Novartis Pharma AG (Basel, Switzerland) by refolding in vitro the monomeric, denatured protein overexpressed in Escherichia coli (14). NMR measurements were performed with a sample of 1.4 mM 15N-labeled TGF-β3 in mixed solvent of 87% H2O, 5% D2O, 6% dioxane-d6 and 2% methanol-d4, at pH 7.8 and 40 °C.

It was shown that these conditions do not affect the TGF-β3 structure in solution, but provide optimal resolution in 1H-15N correlation spectra of TGF-β3 (see Supplementary Material) and fully remove aggregation of TGF-β3, which prevent NMR relaxation analysis (12, 13). In view of the therapeutic potential of TGF-β, we present here a dynamic study of TGF-β3 aimed at explaining its behavior in aqueous solution and characterizing determinants involved in the interaction with receptors.

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relaxation delays ranging from 10 to 1500 ms in $R_2$, and from 0 to 200 ms in $R_1$ experiments. The $R_2$ experiments were carried out using a CPMG sequence with 1.0-ms delay between consecutive 15N 180° pulses. To account for off-resonance effects in CPMG the $R_2$ values were numerically corrected (16). The $^{15}$N[$^1$H] NOE values were calculated as a ratio of signal intensities in spectra recorded with and without prior saturation of amide protons. The delay between scans in the NOE experiment was 6.0 s. The proton saturation in the NOE experiment was achieved by a sequence of 120° [$^1$H] pulses applied during 5.0 s. All spectra were processed and quantified using a macro within the VNMR software. Each of the $R_1$, $R_2$, and NOE experiments were repeated twice, and the data were averaged over the measured data sets. To account for possible small systematic errors in the experimental data, minimal uncertainties of 3% for $R_1$ and $R_2$ and ±0.05 for NOE were assumed for the subsequent “model-free” data analysis. The evidence that TGF-β3 does not aggregate at 1.4 mM concentration was provided by a $R_1$ experiment repeated on a diluted 0.14 mM protein sample.

Hydrodynamic Calculations—For comparison with the experimental data the rotation diffusion tensor $D_{ij}$ of the TGF-β3 dimer was estimated by hydrodynamic calculations using the beads model approximation (17) based on the crystal structure of the TGF-β3 dimer (10) (PDB code 1tgj). Each residue of TGF-β3 was represented by a spherical friction element (bead) of a 3.5-Å radius centered at the position of its Cα atom (18). Hydrodynamic interactions between beads were accounted for by a modified Oseen tensor. The calculations were performed with the DIFFPC program (19).

Model-Free Analysis of the Relaxation Data—Model-free analysis (20, 21) of the relaxation data for TGF-β3 was performed with the DASHA software (19) using a procedure described elsewhere (22, 23). Values of 1.02 Å for the NH intranuclear distance and −170 ppm for the $^{15}$N chemical shift anisotropy were used in the analysis. The extensive set of nine independent relaxation measurements for each NH group allowed us to use the most complex anticipated model-free spectral-density function. Namely, intramolecular motion of an NH group was described by four adjustable parameters of the “extended” reorientation correlation function (24): order parameters $S_x^2$ and $S_y^2$ and correlation times $\tau_1$ and $\tau_2$, of pico- and nanosecond motions, respectively, and an extra parameter $\lambda$ by the adjustable exchange term proportional to the square of the magnetic field strength. Anisotropic rotational diffusion of TGF-β3 was accounted for by five-exponential overall rotation correlation functions of the NH groups (25). The parameters of intramolecular motions of all nuclei were optimized simultaneously with six parameters of anisotropic overall rotation: rotation correlation time $\tau_R$ and principal components of rotational diffusion tensor $D_{ij}$, $D_{Ri}$, and $D_{Rj}$, and the Euler angles $\alpha$, $\beta$, and $\gamma$ defining the orientation of the molecular frame where $D_{Ri}$ has diagonal form with respect to the reference molecular frame. The uncertainties of the optimized parameters were obtained from the covariance matrix of the optimized model (26). The directions of the NH vectors in the reference molecular coordinate frame were taken from the crystal structure of TGF-β3 (10) (PDB code 1tgj).

RESULTS

Experimental Relaxation Data—$^{15}$N relaxation data were obtained for the backbone amides of 86 out of the 103 nonproline residues of TGF-β3 at magnetic fields of 11.7, 14.1, and 18.8 T. The relaxation data for the remaining residues were not extracted due to spectral overlap and/or resonance broadening (especially for the N-terminal residues 6–17). The measured $^{15}$N[$^1$H] NOE, $^{15}$N $R_1$, and $R_2$ values (Fig. 1, a–c) exhibit significant variations along the protein sequence. Namely, residues 2–5, 31, 50–75, 82, and 91–98 with lowered $^{15}$N[$^1$H] NOE and $^{15}$N $R_2$ values appear to exhibit significant pico- to nanosecond time scale motions. Residues 13, 18, 21, 27, 30, 32, 42, 43, 86, and 104 have enhanced $R_2$, indicating micro-millisecond conformational exchange.

Overall Rotation of the TGF-β3 Dimer in Solution—A more rigorous analysis of the relaxation data was performed using the model-free approach (20). The experimental relaxation data were fit by simultaneous optimization of the parameters of anisotropic rotation of TGF-β3 and the parameters of intramolecular motions of individual NH groups for all $^{15}$N nuclei. This procedure yields an overall rotation correlation time $\tau_R$ of 16.0 ± 0.3 ns, which is somewhat higher than the 14.3 ± 0.6 ns calculated from the mean of the $^{15}$N $R_2/R_1$ ratios of all NH groups in regular secondary structure elements with $^{15}$N[$^1$H] NOE values higher than 0.6. Such an underestimation is inherent to $\tau_R$ values derived from $R_2/R_1$ ratios with the assumption of very fast internal dynamics due to the unaccounted contributions of nanosecond time scale motions (22). The ratios of principal components of the rotational diffusion tensor $D_{ij}$ = 0.72 ± 0.14 and $D_{Ri}/D_{Rj}$ = 0.78 ± 0.16 derived from the relaxation data reveal moderate anisotropy of TGF-β3 tumbling in solution. Hydrodynamics calculations based on the crystal structure of the TGF-β3 dimer predicts similar rotation diffusion tensor although with more pronounced anisotropy: $D_{ij}$ = 0.42 and $D_{Ri}/D_{Rj}$ = 0.45. These results suggest a less extended fold of the TGF-β3 dimer in solution than in crystal, probably due to less compact packing of regular structure elements. This is in agreement with our previous NMR study (13), which showed that the secondary structure and the global fold of the TGF-β3 dimer in solution are close to those obtained for the protein crystal, but some regions are in dynamic equilibrium with an unfolded conformation. Moreover the CD studies in aqueous solution show that the α-helical content of TGF-β3 in solution is less then expected from the known crystal and solution structures of TGF-β3, including free TGF-β3 in crystal (12). In contrast, a good agreement between the experimental CD spectrum measured in aqueous solution at pH 3.0 and CD spectrum calculated from crystal structure was found for TGF-β2 (12).

Internal Dynamics of the TGF-β3 Dimer—Model-free analysis of the relaxation data yields the order parameters $S_x^2$ and $S_y^2$ describing motions in the picosecond and nanosecond time scales, respectively, and the corresponding correlation times $\tau_1$ and $\tau_2$ (Fig. 1, d–f). The generalized order parameter $S^2 = S_x^2 + S_y^2$, plotted in Fig. 1d, characterizes therefore fast motions, in contrast to exchange contribution $R_{ex}$ (Fig. 1g) to $R_2$ and resonance broadening, which indicate slow motions in the micro-millisecond time range. The generalized order parameter $S^2$ provides a measure of the amplitude of internal motion, where $S^2 = 1$ means that the given N-H bond vector is fixed on the pico- to nanosecond time scale, and $S^2 = 0$ indicates that the motion is unrestricted. These internal dynamic properties derived from the relaxation analysis are summarized in Fig. 2 by color-coded ribbon diagrams of the TGF-β3 crystal structure (10). Fig. 2a summarizes fast motions by mapping of the generalized order parameter $S^2$ to the three-dimensional structure, while Fig. 2b visualizes regions with slow motions. Several regions of TGF-β3 displaying different types of internal motions of various amplitudes and time scales, from fast pico- to nanosecond dynamics to slow conformational transitions on micro-millisecond and second time scales, were identified and are characterized below.

Each monomer of the TGF-β3 dimer adopts an extended fold made up of four helices and four β-strands arranged in two irregular antiparallel β-sheets, βA/βB and βC/βD (see Fig. 3c) (10, 13). The β-sheets represent the most rigid part of TGF-β3 having high order parameters. Slightly enhanced pico- to nanosecond mobility with lowered $S^2$ values is observed in the vicinity of the short breaks of both β-sheets located at residues 19–20/41–42 and 81–82/107–108, especially for residues 18, 21, 41, 82, and 107. Additionally, the spatially adjacent segments 18–21/40–43 and 86–87/102–104, which are close to the break and twist of the first and the second β-sheets, respectively, are involved in micro-millisecond motion as revealed by $R_{ex}$ and resonance broadening observed for the residues from the first β-sheet. Residues 77 and 79 in the hydrophobic dimer interface and residues 34 and 90 in the hydrophobic pocket near the first β-sheet loop are also affected by slow conformational exchange. The largely remote region 91–97 comprising
the backbone $^{15}$N nuclei of TGF-$\alpha$ spectrometers, are shown in FIG. 1.}

Experimental relaxation data for residues 24–28 and an extended loop 29–32. Note that the loop 29–32 is also flexible on the pico- to nanosecond time scale revealed by lowered order parameters for residues 29 and 31.

The most flexible regions of the molecule are the N-terminal residues 2–5 and the region between the $\beta$-sheet strands $\beta B$ and $\beta C$ with residues 51–75 comprising the central $\alpha$-helix $H 3$ and the one-turn $3_{10}$-helix $H 4$. In this region, the low and non-uniform order parameters $S_{\tau}^2$ and $S_{\tau}^2$ and the high $\tau_2$ of 40–70 ps suggest a wide range of processes occurring in the pico- to nanosecond time scale presumably connected with temporary ruptures of the helices $H 3$ and $H 4$. The helix-coil equilibrium in TGF-$\beta 3$ was previously suggested based on the chemical shift indices of $^1$Haa and on $^1$H-NOE connectivities (13). Additionally, the region 48–75 is involved in another slow process resulting in disproportional doubling and/or tailing of the amide resonances. This process likely occurs on the second time scale with characteristic times slower than $^{15}$N $T_1$ revealed by the absence of detectable exchange cross peaks in ZZ-exchange spectra (27). A possible source of this slow exchange is cis-trans isomerization of the Cys$^{40}$-Pro$^{49}$ and/or Ser$^{75}$-Pro$^{76}$ peptide bonds.

**DISCUSSION**

**Dynamic Mapping the TGF-$\beta 3$ Receptor Recognition Sites**—The members of the TGF-$\beta$ superfamily mediate their functions by binding to cell surface receptors. Several functional regions in TGF-$\beta$ were mapped using site-directed mutagenesis of TGF-$\beta 1$ and domain swap approach applied to TGF-$\beta 1$/$\beta 2$ chimera (28, 29). It was shown that region 52–55 is very important for the interaction of TGF-$\beta$ with the cell receptors, whereas region 92–98 regulates specific binding of isomers to T$\beta 2$ only. Lately, for BMP-2 (6), activin A (30), and glial cell-line derived neurotrophic factor (31), i.e. other members of the TGF-$\beta$ superfamily, mutant proteins that exhibit altered biological activity and receptor binding affinity were constructed and analyzed. Furthermore, the crystal structure of BMP-2 in complex with two ectodomains of its type I receptor (ecBRI1a-BMP-2) and the recently obtained crystal structure of TGF-$\beta 3$ in complex with two ectodomains of its type II receptor (ecT$\beta R 2_c$-TGF-$\beta 3$) now serve as the prototypes for the intermediate complexes of the TGF-$\beta$ superfamily (32, 33). Comparison of the ecT$\beta R 2_c$-TGF-$\beta 3$ interface with either the state $^{15}$N/$^1$H NOE, b, $^{15}$N longitudinal relaxation rate $R_2$; c, $^{15}$N transverse relaxation rate $R_2$. Dynamics parameters of individual NH groups of TGF-$\beta 3$, obtained in model-free analysis, are shown in d–g: d, the order parameters $S_{\tau}^2$ (green) and $S_{\tau}^2$ (red) of pico- and nanosecond time scale motions, respectively, and generalized order parameters $S_{\tau}^2 = S_{\tau}^2 S_{\tau}^2$ (black); e and f, the correlation times $\tau_2$ and $\tau_2$ of pico- and nanosecond time scale motions, respectively; g, exchange contribution $R_{ex}$ to $R_2$ quantified at 11.7 T. The $R_{ex}$ contributions are very similar to those (see Supplementary Material) estimated using field dependence of $R_2$ (54). The uncertainties are shown by bars. Broken lines indicate the region 6–17, which is poorly sampled due to strong broadening of NMR signals.
activin-binding site on ecActR2 or the ecBR2-binding site on BMP-2 suggests that there is significant structural diversity in the manner by which type II receptors of the TGF-β superfamily interact with their cognate ligands (33). As a result, two epitopes designated as “wrist” and “knuckle” interacting with BMP type I and type II receptors, respectively, were found and mapped onto the spatial structure of BMP-2. In addition the “fingertips” epitope recognizing the TGF-β type II receptor was found in the TGF-β ligand. Overall, these findings provide a framework for the molecular description of receptor recognition and activation in the TGF-β superfamily.

The monomer fold of the TGF-β dimer was described as an
TGF-β3 shows an altered arrangement of its monomeric subunits (33). Assuming that the backbone mobility of TGF-β3 active sites is reduced upon receptor binding, one would expect a strongly unfavorable entropic contribution to the free energy of complex formation (34). All sources of changes in free energy are critical in the delicate energy balance that determines the equilibrium populations of protein-ligand or protein-protein interactions. This implies that the loss of conformational entropy due to the ordering of TGF-β3 binding surface must be efficiently compensated by hydrophobic interactions and enthalpic factors such as H-bond or electrostatic interactions. Furthermore, the enhancement of TGF-β3 internal mobility tends to cluster at both epitopes that may indicate localized “hot spots” (35) of binding free energy.

The hairpin loop region 92–95, the one-turn α-helix H2, and the extended loop 29–32 are suitable for such hot spots in the fingertips epitope of TGF-β3. The residues Arg95 and Arg32 from these regions play an important role in determining binding specificity and affinity to TβR2 (29, 33). Several potential hot spots of binding free energy are observed in the more spacious wrist epitope putative for recognizing the TGF-β type I receptor. The most prominent of these is the central α-helix H3 with its pre-helix loop identified as an active site by mutagenesis analysis (28) of TGF-β1. Another candidate common with the fingertips epitope is region 29–32 situated in a hydrophobic pocket nearby the outer part of the β-sheet loops. This pocket in TGF-β3 can accommodate a dioxane molecule both in crystal (10) and in solution (13). Furthermore, in the BRIAl2-BMP-2 complex this hydrophobic pocket interacts with the side chain of Phe84 of BRIA corresponding to Phe84 of TβR1. That is perhaps a key feature for the recognition of type I receptor within the TGF-β superfamily (32). The next possible hot spot of binding free energy is the N-terminal part of TGF-β3, designated as thumb epitope, which is spatially close to wrist epitope and contains many non-conservative residues in the solvent-exposed flexible loop following α-helix H1 (Fig. 3c). This segment is found only among the TGF-β3 and the activins but not in other members of the TGF-β superfamily and may also participate in the TGF-β recognition of type I receptor. Thus, the low affinity wrist epitope of TGF-β3 is more flexible in comparison with the fingertips epitope that apparently results in the most pronounced conformational entropy loss upon receptor binding. In the vacant knuckle epitope of TGF-β3 two interesting dynamic clusters adjacent to the fingertips and thumb epitope may be identified as potential hot spots of binding free energy. The first is the region 34–37 from the N-terminal part of β-strand βB, which partially participates in the interaction with eTβR2. The second is the hydrophobic patch nearby the break of the first β-sheet, containing residues Tyr71 and Tyr80. These hot spots may serve to recognizing other TGF-β-binding proteins, such as betaglycan. Betaglycan has two binding sites for TGF-β and plays an important role in assembling a potentially more productive TGF-β-receptor signaling complex (36). Moreover TGF-β-binding sites to different proteins probably overlap, and described above hot spots in the TGF-β epitope may also interact with other TGF-β activity regulation proteins such as LAP and thrombospondin. LAP binding to TGF-β inhibits the ligand binding to receptor sites on the TβR2 and betaglycan (36). Thrombospondin binds to a site in the active domain of TGF-β through three repeats of hydrophobic WXXW motif (3), which are known to become posttranslational C-mannosylated (37). As shown previously the hydrophobic pocket close to the fingertips epitope is potentially capable of binding a carbohydrate ring (10) that suggests the thrombospondinin-binding site in the TGF-β molecule. The suggestions discussed above are based on the well known fact...

outstretched “hand” with the N-terminal α-helix H1 as the “thumb” and the extended sheets as slightly curled “fingers” (9, 10) (Fig. 2a). It was determined that the residues of the loops at the tips of both β-sheets form the high affinity fingertips epitope (33). The wrist epitope comprises residues of α-helix H3 and its pre-helix loop of one subunit and contacts both the inner side of the loop at the tip of the first β-sheet and the C-terminal part of β-strand βC of the other subunit of the dimer (32). In turn the putative knuckle epitope mapped (6) onto the outer convex surfaces of both β-sheets is spatially adjacent to the fingertips epitope and oppose the wrist epitope.

The color-coded ribbon diagrams (Fig. 2, a and b) demonstrate that the most dynamic regions of the TGF-β3 dimer exactly correspond to the receptor-binding sites of the fingertips as well as wrist epitopes in contrast to the knuckle epitope. For clarity we constructed the model of TGF-β3-receptor signaling complex based on the crystal structures of ecBRIA2-BMP2 (32) and ecTβR2,TGF-β3 (33) complexes (Fig. 2c). Interestingly the interface between subunits of free TGF-β3 is flexible, which suggests the possibility of the structural accommodation of the TGF-β3 dimeric fold upon receptor binding. This is consistent with the fact that ecTβR2-bound TGF-β3 shows an altered arrangement of its monomeric subunits (33). Assuming that the backbone mobility of TGF-β3 active sites is reduced upon receptor binding, one would expect a strongly unfavorable entropic contribution to the free energy of complex formation (34). All sources of changes in free energy are critical in the delicate energy balance that determines the equilibrium populations of protein-ligand or protein-protein interactions. This implies that the loss of conformational entropy due to the ordering of TGF-β3 binding surface must be efficiently compensated by hydrophobic interactions and enthalpic factors such as H-bond or electrostatic interactions. Furthermore, the enhancement of TGF-β3 internal mobility tends to cluster at both epitopes that may indicate localized “hot spots” (35) of binding free energy.

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that intrinsically unstructured proteins offer important advantages in cellular signaling and regulation. Their inherent flexibility allows their local and global structure to be modified in response to different molecular targets, allowing one protein to interact with multiple cellular partners and allowing fine control over binding affinity (38).

Comparison of Internal Dynamics for TGF-β Isoforms—The results presented above and literature data reveal that TGF-β dynamics are different from that of other TGF-β isoforms. In Fig. 3a the backbone $^{15}$N{1H} NOEs of TGF-β3 obtained in the present study are plotted versus $^{15}$N{1H} NOEs of TGF-β1. This plot is chosen for comparison, since the order parameters $S_2$ were not reported in the NMR study of TGF-β1 in solution (11). The lowered $^{15}$N{1H} NOEs clearly indicate the regions with enhanced mobility on the subnanosecond time scale. Although direct comparison of NMR and x-ray data on protein dynamics may be considered problematic (39, 40), in Fig. 3b we also list, as a reference information, the thermal B-factors taken from crystallographic studies of TGF-β3 (9, 10, 33) reflecting the local mobility of a protein constrained by a crystal lattice. Thermal B-factors of free TGF-β2 and free TGF-β3 suggest that the β-sheets and the helix H3 represent the most rigid parts of these TGF-β isoforms, while internal mobility is increased for loop regions connecting secondary structure elements. The $^{15}$N NMR relaxation study of TGF-β1 suggests an overall rather restricted pico- to nanosecond mobility for the protein with somewhat more flexible regions 2–4, 10–13, 50–56, 71–75, and 91–98 (11). The same regions of TGF-β3 in solution also exhibit internal motions over a wide range of time scales. However, there are several differences in internal dynamics of TGF-β3 in solution from that anticipated from previous studies of TGF-β isoforms. The major difference is that in TGF-β3 the central α-helix H3 and the C-terminal part of its pre-helix loop are extremely flexible in solution. Note that this region is the least conserved among the TGF-βs (Fig. 3c). Transient disruptions of helical structure in TGF-β3 are presumably caused by the presence of four helix destabilizing residues (Thr$^{57}$, Thr$^{60}$, Gly$^{63}$, and Thr$^{67}$) on the outer face of the α-helix H3 alongside its pre-helix loop. Similarly, the regions 1–12 and 55–72 of ecfTβ2-bound TGF-β3 are also disordered in the crystal (33). In contrast, the crystal structure of free TGF-β3 displays low thermal B-factors and well defined electron density throughout α-helix H3. Crystal packing forces are responsible for such ordering, as several residues of this segment participate in intermolecular interactions in the crystal lattice (10). These observations emphasize the importance of the NMR information on protein dynamics in solution for this family of proteins.

Relationship between the Unique Dynamics and Specific Macroscopic Behavior of TGF-β3—Our results indicate that TGF-β3 is significantly more flexible than other proteins of this family. Thus, certain protein properties should be very sensitive to mild variations of local environment. The high backbone flexibility is related to the local unfolding of the protein. This, in turn, results in exposure of the hydrophobic regions and may cause aggregation. As shown previously, the association of oligomeric proteins is generally an entropy-driven process (41). Although TGF-β3 does not form aggregates under the conditions of the experiments (pH 2.9), the flexible nature of the molecule is correlated with the tendency to aggregate and bind to surfaces at higher pH (12). In contrast, TGF-β1 and TGF-β2 easily solubilize in aqueous solutions at physiological pH. All that allows us to propose that the unusual dynamics of TGF-β3 is related to its specific macroscopic behavior and biological activity. The good solubility of TGF-β1 and TGF-β2 at physiological conditions may be associated with the significantly elevated plasma levels of TGF-β1 and TGF-β2, but not of TGF-β3 in patients with disseminated malignant melanoma (42). TGF-β3 is possibly deposited in its aggregated form and stays at its place of secretion. The poor solubility of TGF-β3 between pH 5 and pH 8 and its good solubility below pH 4 could explain localized biological activity of the molecule in processes like bone repair. Local bone remodeling involves low pH digestion of the bone by osteoclasts at the ruffled border. Bone proteins, including TGF-β, are released from the bone by osteoclasts in the ruffled border compartment (pH < 4) and presumably transcytosed to the osteoclasts apical surface (43, 44). The growth factors activated by low pH exposure can locally stimulate osteoblasts and contribute to the formation of new bone matrix. The fact that flexibility and aggregation of TGF-β3 are likely correlated with its function (the growth factor should act close to the place of secretion) has the implication that this protein is beneficial in local therapies by remaining confined at the application site.

Concluding Remarks—The study presented here shows that highly dynamic regions of TGF-β3 cluster at the receptor-binding epitopes. This manifests an important role of internal dynamics in providing the specificity and affinity of TGF-β3 to receptors. Moreover, the differences in internal dynamics of TGF-β isoforms provide the basis for an explanation of the variations in their macroscopic behavior and interactions with receptors. Those are related to the control of TGF-β release and activity, which are critical in many diseased states and injury repair processes. This once again confirms that function (and thus potential clinical application) cannot be based on the knowledge of spatial structures alone, but that dynamic aspects are of utmost importance.

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