Annu. Rev. Biophys. Biomol. Struct. 1995. 24:269–91

THE CYSTINE-KNOT GROWTH-FACTOR SUPERFAMILY[1]

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KEY WORDS: X-ray structure; common cytokine fold; comparison of TGF-β, PDGF, NGF, and hCG; disulfide motif

CONTENTS

ABSTRACT

Four recent crystal structures of growth factors—nerve growth factor, transforming growth factor-β, platelet-derived growth factor, and human chorionic gonadotropin—from four separate superfamilies revealed that these proteins are structurally related and share a common overall topology. These proteins have very little sequence homology, but they all have an unusual arrangement of six cysteines linked to form a "cystine-knot" conformation. The active forms of these proteins are dimers, either homo- or heterodimers. Despite the overall topological similarity between the monomers, the interfaces used to form the dimer are in each case quite different. Because the surfaces used for dimer formation are mostly hydrophobic, the uniqueness of each dimer accounts for the lack of sequence homology and raises questions about the effectiveness of reverse sequence fitting in this kind of structure as a predictor of structural homology.

INTRODUCTION

Growth factors compose a group of diverse protein molecules that regulate cell growth, differentiation, and cell-cell communications. Although the molecular mechanisms that govern growth factor–mediated processes remain largely unknown, growth factors can be generally classified into several superfamilies based on their structural and functional similarities. Examples of these superfamilies include: (*a*) the hematopoietic growth factors, such as growth hormone, IL-2, IL-4, G-CSF, and CNTF, which all possess a four-helix-bundle structural motif (10, 26, 94); (*b*) the β-trefoil family members, such as IL-1β, IL-lα, FGF, and keratinocyte growth factor, which all share a common β-trefoil fold (28, 91, 99); (*c*) the EGF-like growth factors such as EGF and TGF-α, which all have an immunoglobulin-like domain in their structure (66).

The number of new growth factor structures available is increasing, and a new growth-factor fold, the so-called cystine-knot growth-factor fold, has unexpectedly emerged. NGF, TGF-β, PDGF, and glycoprotein hormones serve as prototypes of this superfamily (22, 60, 67, 68, 86). Unlike other growth factor families, the protomer structures of all members of this fold have a cystine-knot motif located in topologically equivalent positions, but otherwise, they share no significant sequence homology. As a result, it is often difficult to recognize a member of this superfamily before its three-dimensional (3D) structure is known. For the same reason, this is the only superfamily in which the knowl-

edge of a 3D structure becomes the most definite and sometimes the only way to define a member. Although they bind to quite different receptor families, the remarkable structural similarity between these growth factors suggests that they have probably evolved from a common ancestor. Another unique feature of this superfamily is that they all function in a dimeric form, and sometimes both hetero- and homodimers can be formed.

In this review, we summarize the structural and functional properties of this superfamily and compare the crystal structures of four of the family's prototypes, TGF-β, NGF, PDGF, and hCG.

BIOLOGICAL FUNCTIONS

TGF-β Family

The TGF-β family consists of a set of growth factors that share at least 25% sequence identity in their mature amino acid sequence. Members in this gene family include the transforming growth factors, TGF-β1–5 (4, 19, 27, 40, 41, 45, 87); inhibins and activins (inhibin A, inhibin B, activin A, and activin AB) (30, 53, 56, 89); bone morphogenic proteins, BMP-2–7 (17, 71, 95); the decapentaplegic gene complex, DPP-C (72); Vgl (93); vgr-1 (55); Müllerian inhibiting substance, MIS (16); a growth-differentiation factor, GDF-1 (49); and dorsalin-1, dsl-1 (8). Most of these factors exist as either homo- or heterodimers (Table 1).

The best-studied factors of this gene family are the transforming growth factors. They form homodimeric proteins with a molecular weight of 25,000. Five isoforms of TGF-βs have been isolated to date from different species, and the mature peptide sequences of these proteins share 64–82% homology. TGF-β1–3 are found in mammalian cells, TGF-β3 and 4 in chicken embryos, and TGF-β5 in *Xenopus laevis* (57, 85). They can be found in virtually any cell type and throughout the developmental stages of any given species.

The diverse biological activities of TGF-β in cell growth and regulation can be classified as: (*a*) effects on the cell cycle, (*b*) effects on the extracellular matrix, and (*c*) effects on other peptide growth factors. Many of the cell growth–inhibitory effects of TGF-β directly result from its ability to interrupt the cell cycle during late G_1 phase, preventing induction of DNA synthesis and progression into S phase. For example, TGF-β1 exhibits potent growth- and differentiation-inhibitory effects in many endothelial and epithelial cells and is a negative regulator during myogenesis (88), osteogenesis (18), and embryogenesis (35). In mesenchymal cells, TGF-β increases cell accumulation and their

Table 1 Members of the cystine knot growth factor superfamily

Family	Active form	Family	Active form
TGF-β		PDGF	
TGF-β1–5	Homodimer	PDGF-AA	Homodimer
Inhibin A	α,βA dimer	PDGF-AB	Heterodimer
Inhibin B	α,βB dimer	PDGF-BB	Homodimer
Activin A	βA homodimer	*v-sis*	Homodimer
Activn AB	βA,βB dimer	VEGF	Homodimer
BMP-2,3,4,5,6,7	Homo- or heterodimer		
DPP-C		Glycoprotein hormone	
Vg1		hCG	α,β dimer
vgr-1		LH	α,β dimer
GDF-1		FSH	α,β dimer
dsl-1		TSH	α,β dimer
MIS	Homodimer		
Neurotrophin			
NGF	Homodimer		
BDNF	Homodimer		
NT-3,4	Homodimer		

response to extracellular-matrix components, including type I, III, IV, and V collagen; tenascin; and elastin (54, 73, 90). TGF-β can also promote or inhibit cell growth by modulating the secretion of other growth factors. For example, it stimulates the growth of fibroblasts by increasing the level of PDGF secretion (78).

Three types of TGF-β receptors, I, II, and III, are expressed on the cell surface in order to mediate the growth-factor signal (100–103). Types I and II are members of the Ser/
whereas the type III receptor, a proteoglycan whose function is to present TGF-βs to the type I and II receptors, does not have a functional cytoplasmic domain.

Neurotrophic Factors

The neurotrophins represent a family of growth factors that control the development and survival of certain neurons in both the peripheral (PNS) and the central nervous systems (CNS). The members of this family include nerve growth factor (NGF) (51), brain-derived neurotrophic factor (BDNF) (32, 50), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and neurotrophin-5 (NT-5) (6, 13, 33). Among them, NGF is the prototypical neurotrophin that defines the properties and functions of this class of growth factors.

NGF is synthesized and released from target tissues in both the PNS and CNS. In the PNS, the target tissues are typically nonneuronal cells while in the CNS the targets are neurons such as the sympathetic, sensory, and cholinergic basal forebrain neurons (52). The role of NGF as a survival reagent during nerve development comes mainly from its ability to rescue some neurons from naturally occurring cell death, thus preventing nerve degeneration (7). In adult tissues, the function of NGF is less well understood.

There are two different classes of neurotrophin receptors, a low-affinity receptor, $p75^{NGFR}$, which serves as a common receptor for all the known neurotrophins, and a high affinity receptor, $p140^{trk}$, which belongs to the trk family of tyrosine kinase receptors and is different for each neurotrophic factor (43, 62).

PDGF Family

Platelet-derived growth factor (PDGF) is a major mitogenic factor for cells of mesenchymal origin. It promotes the growth and differentiation of fibroblasts and smooth muscle cells during development and embryogenesis. It also functions as a chemotactic reagent for inflammatory cells during wound healing (36). Two forms of the PDGF gene are expressed, PDGF-A and PDGF-B, resulting in three isoforms of the dimeric growth factor, PDGF-AA, PDGF-AB, and PDGF-BB. All three have similar biological functions as cell mitogens, but they differ in the activation of the receptor isoforms. Like the growth factor itself, the gene for the receptor also takes two forms, PDGF-α and -β receptors (58, 82), which results in three functional dimeric receptor isoforms, ⟨aa⟩, ⟨ab⟩, and ⟨bb⟩. Both forms of the receptor have an intracellular tyrosine kinase domain, but they respond differently to the different isoforms of PDGF (12, 37): PDGF-AA only complexes with the ⟨aa⟩ receptor; PDGF-AB complexes with the ⟨aa⟩ or ⟨ab⟩ receptor; and PDGF-BB can complex with all three receptor isoforms, ⟨aa⟩, ⟨ab⟩, and ⟨bb⟩ (34, 84).

Other members the PDGF family include the vascular endothelial growth factor (VEGF) and the *v-sis* oncogene product of $p28^{v\text{-}sis}$, a transforming protein of simian sarcoma virus (SSV). The $p28^{v\text{-}sis}$ sequence is 90% homologous to that of PDGF-B and 50% homologous to PDGF-A (11, 77). It binds to and activates both the α and β PDGF receptors (48).

Glycoprotein Hormones

Glycoprotein hormones refer to a set of four related glycopeptides: human chorionic gonadotropin (hCG), follicle-stimulating hormone

(FSH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH). They are dimers of two noncovalently linked α- and β-subunits (69, 75). All four glycoprotein hormones share a common α-subunit, whereas each has its unique β-subunit that determines its specific hormone activity (14, 29). hCG is thought to maintain the early stages of pregnancy, acting to prolong corpus luteum function. In mammalian systems, LH and hCG bind to the same receptor and possess similar biological functions, whereas FSH and TSH bind to structurally similar but distinct receptors. Interestingly, the level of FSH in pituitary cells and the level of hCG in human placenta cells are modulated by the inhibins and activins, members of the TGF-β family (30, 56).

THREE-DIMENSIONAL STRUCTURE DETERMINATIONS

Human Transforming Growth Factor β2 (TGF-β2)

Daopin et al (25) and Schlunegger & Grütter (80) simultaneously reported the crystal structure of TGF-β2 at 2.1 and 2.2 Å resolution, respectively. Both groups crystallized the mature form of the protein, 112 amino acids, in a space group of $P3_221$ with the cell dimensions $a = b = 60.6$ and $c = 75.3$ Å. Later, structural comparison showed that the two structures are essentially identical (23). The structure from Davies' group was then refined to 1.8-Å resolution (24) and that from Grütter's group was refined to 1.95-Å resolution (81). At about the same time, an NMR characterization of the secondary structure of TGF-β1 was also reported, and the predicted tertiary NOEs were in agreement with the crystal structure obtained from Davies' group (2, 3).

The structure shows TGF-β2 as a disulfide-linked dimer in which the overall dimensions of each monomer are $60 \times 20 \times 15$ Å (Figure 1*a*). The secondary structure of the protomer consists mainly of four irregular antiparallel β-strands (labeled as 1, 2, 3, and 4 in Figure 1*a*) and an 11-residue α-helix between the second and the third strand. Of the nine cystines in each monomer, eight of them form four intrachain disulfides, and one, cystine 77, forms an interchain disulfide with the same cystine of another monomer. The four intrachain disulfide bonds are between Cys7 and Cys16, Cys15 and Cys78, Cys44 and Cys109, and Cys48 and

Figure 1 Ribbon diagrams of the 3D structures. The two monomers are shaded differently and the knotted disulfides are shown in solid bonds. (*A*) TGF-β2; (*B*) β-NGF; (*C*) PDGF-BB; (*D*) hCG-α (*shaded subunit*) and hCG-β. Molscript was used to prepare the figure (46).

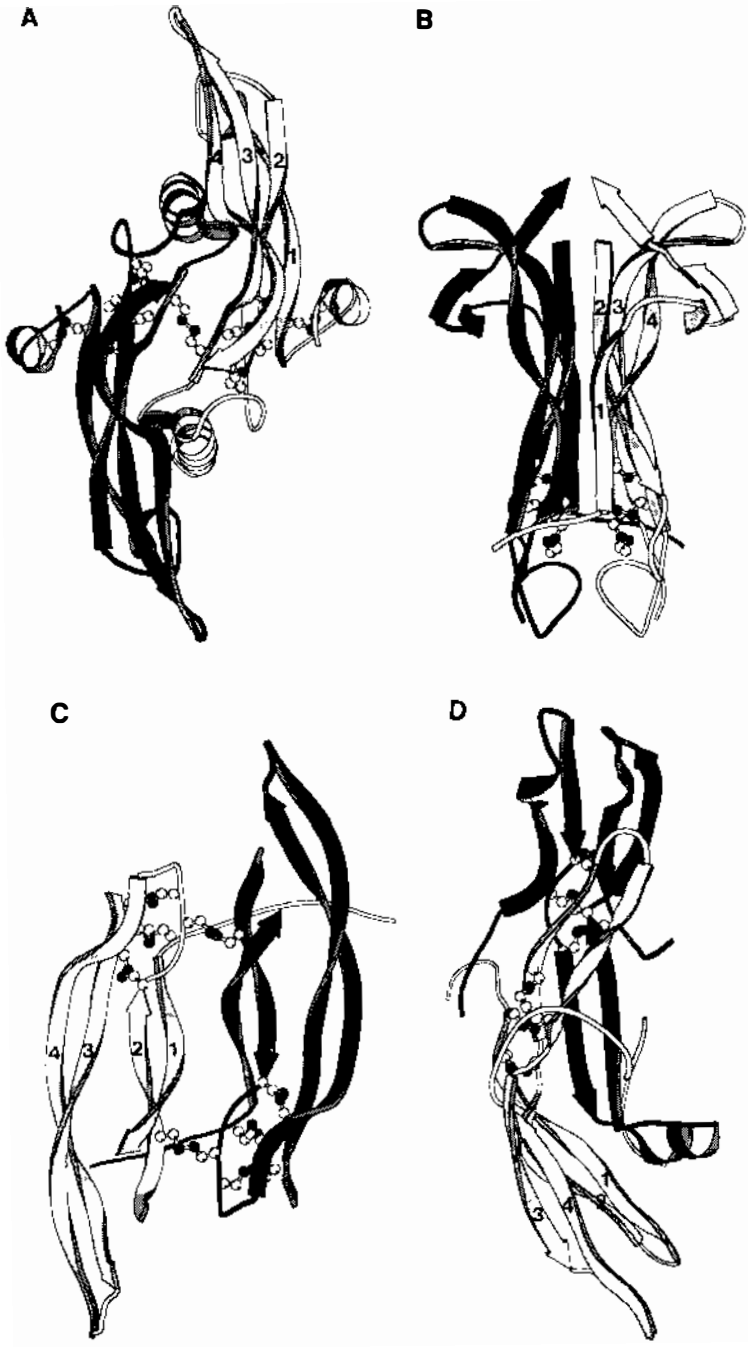

Table 2 List of disulfide bonds

Cystine knot	β-NGF	TGF-β2	PDGF-BB	hCG-α	hCG-β
I–IV	15–80	15–78	16–60	10–60	9–57
II–V	58–108	44–109	49–97	28–82	34–88
III–VI	68–110	48–111	53–99	32–84	38–90
Interchain	None	77–77	43–52		
			52–43		
Other		7–16		7–31	23–72
				59–87	26–110
					93–100

Cys111. The latter three, Cys15-Cys78, Cys44-Cys109, and Cys48-Cys111, define a topological cystine knot in which the Cys15-Cys78 disulfide passes through a ring bounded by the Cys44-Cys109 and Cys48-Cys111 disulfides together with the connecting polypeptide backbone, residues 44–48 and 109–111 (Table 2).

The two monomers form a head-to-tail dimer with the residues on the long helix (residues 58–68) packed against the residues near the end of the β-sheets. The molecular interface between the dimer of TGF-β2 is largely hydrophobic, burying a total area of 2600 Å^2.

Murine Nerve Growth Factor (β-NGF)

McDonald et al (59) and Holland et al (39) determined the crystal structure of β-NGF, the first structure found of the cystine-knot family of growth factors. The crystals from McDonald et al belonged to the space group $P6_522$ and had the unit cell parameters $a = b = 56.48$ Å and $c = 182.39$ Å and one monomer in each asymmetric unit (59). The structure was determined at 2.3-Å resolution (Figure 1*b*). The protomer structure consists mainly of four irregular antiparallel β-strands (labeled as 1, 2, 3, and 4 in Figure 1*b*) with an insertion of two shorter strands between the first and the second strand. The overall dimension of the molecule is roughly 60 × 25 × 15 Å. Six cystines in each monomer form the knotted disulfide bonds (Cys15-Cys80, Cys58-Cys108, and Cys68-Cys110) clustered at the one end of all the β-strands. The dimer is formed between the two flat faces of the four-stranded β-sheets, burying a total of 2300 Å^2 of surface area. The interface again is characterized as largely hydrophobic. In the work of Holland et al, five crystallographically unique monomers were present and solved in two different space groups (39). Although the core of the molecule (the disulfide knot and the four β-strands) was nearly identical in all five copies,

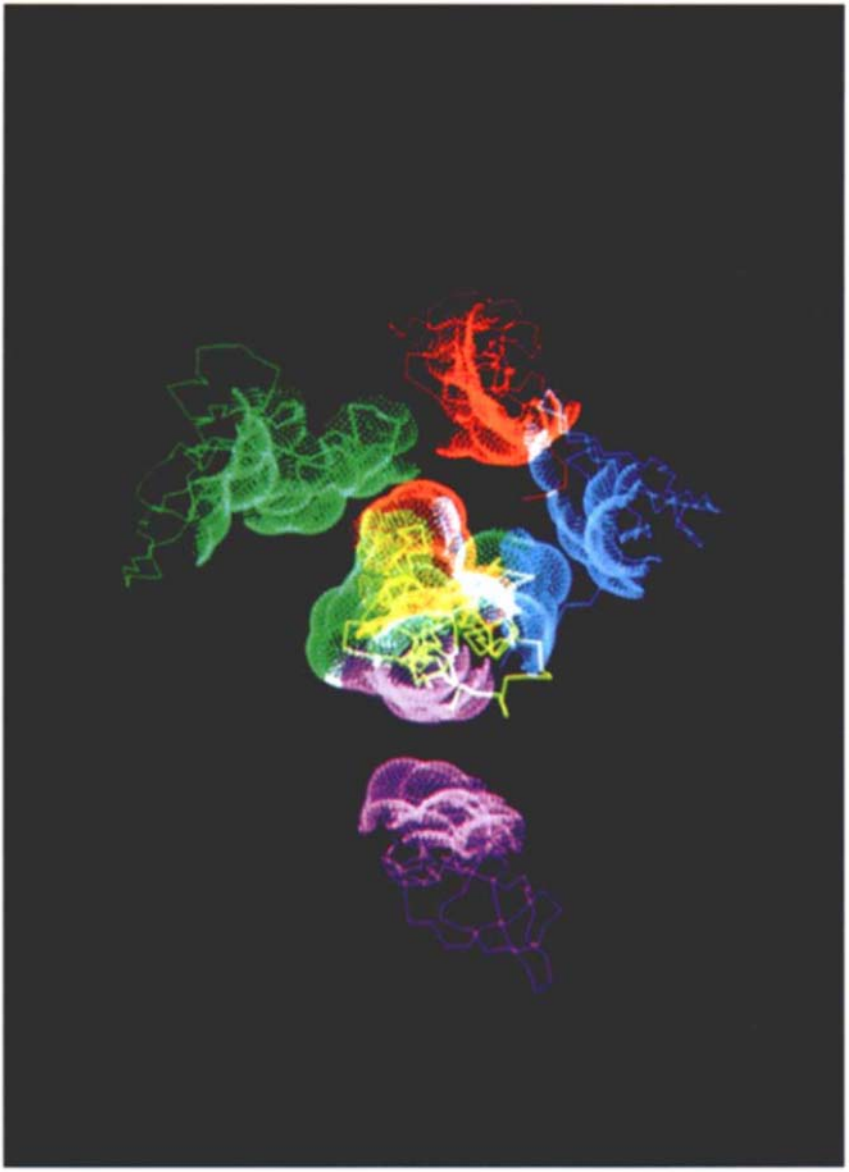

Figure 5 The dimerization modes of the four growth factors. Shown in the center is a generic monomer and surrounding it are the monomers of the TGF-β2 (*green*), β-NGF (*purple*), PDGF-BB (*blue*), and hCG-α (*orange*), which are in their dimerization orientations with respect to the monomer at the center. The dotted surfaces indicate the dimer contact surfaces color coded for each dimer pair. The dimer interface of hCG partially overlaps with that of TGF-β2 and PDGF-BB.

significant flexibility was observed in the loop regions between the four β-strands.

Human Platelet-Derived Growth Factor BB (PDGF-BB)

Oefner et al (70) determined the crystal structure of the mature homodimeric isoform of human platelet-derived growth factor, PDGF-BB, at 3.0-Å resolution. The crystals belong to space group C2 with unit cell dimensions $a = 147.3$ Å, $b = 31.8$ Å, $c = 90.1$ Å, and $\beta = 98.7°$. The protomer has 109 amino acids and consists of four irregular antiparallel β-strands and a 17-residue N-terminal tail (Figure 1*c*). Of the eight disulfide-bonded cysteines, six, Cys16-Cys60, Cys49-Cys97, and Cys53-Cys99, form the knotted arrangement and two, Cys43-Cys52, form two interchain disulfide bonds (Table 2). The edges of the four-stranded β-sheet form the dimer, which results in the majority of intersubunit contacts being between the first two strands of the β-sheet and with the N-terminal tail. The total surface area buried is estimated to be 2200 Å^2, and most of the buried residues are hydrophobic in nature.

Human Chorionic Gonadotropin (hCG)

The most recent addition to the cystine-knot superfamily structures is the determination of the crystal structure of human chorionic gonadotropin by Lapthorn et al (47) and Wu et al (97). Unlike NGF, TGF-β, and PDGF-BB, which are homodimers, hCG is a heterodimer (Figure 1*d*). The α-subunit contains five disulfide bonds, three of which, Cys10-Cys60, Cys28-Cys82, and Cys32-Cys84, adopt the knotted configuration (Table 2). Except for a short three-turn α-helix located between residues 40 and 47, most of the secondary structures in the α-subunit are irregular β-strands and β-hairpin loops. The β-subunit contains six disulfide bonds; among them, Cys9-Cys57, Cys34-Cys88, and Cys38-Cys90 form the topological cystine knot.

Two unique features have been observed in the dimer formation between the α- and β-subunits. First, the segments of well-defined β-sheet structure near the cystine knot in each subunit are brought together upon dimerization to form a short seven-stranded β-barrel, resulting in an interface that has both hydrophobic and hydrogen-bonding interactions. Second, the so-called seat-belt loop, from Cys90 to Cys110 of the β-subunit, is wrapped around the midsection of the α-subunit in the heterodimer to increase the contact surface. The dimerization buries a total of 4525 Å^2 of surface area, according to Lapthorn et al (47), and 3860 Å^2, according to Wu et al (97).

STRUCTURE COMPARISONS

Comparison of the Protomer Structures

Because of the lack of sequence homology, the structural similarity among the four growth factors was not predicted prior to the solution of the 3D structures. It is now clear that the protomer structures of all four families of growth factors share a common fold.

NONGLOBULAR SHAPE OF THE PROTOMER STRUCTURES Each protomer assumes a curled sheet-like nonglobular structure with overall dimensions of approximately 60 × 20 × 15 Å. As a result, they lack a well-defined hydrophobic core. The face of the sheet is formed by four irregular antiparallel β-strands, and near one end of the strands, the most rigid and least exposed part of the molecule, lies a cluster of disulfide bonds in the topological knotted configuration shown in Figure 2. Comparison of the cystines of the knot, for example TGF-β2 and NGF (22), clearly shows that not only are the connectivities of these half cystines identical among all four structures, but their positions are also readily superimposable, resulting in a root-mean-square (rms) agreement of 0.5–1.5 Å between different growth factors for all the atoms in the six half cystines (Table 3, upper diagonal).

COMMON MOTIFS IN SEQUENCE As also noted by McDonald & Hendrickson (60) and Murry-Rust et al (67), some common patterns emerge from the sequence alignment provided by the structural superpositions: (*a*) The spacing of the last two cystines is always CXC—with only one residue between CysV and CysVI. (*b*) The size of the cystine ring depends on the spacing between CysII and CysIII, which varies from 3 to 15. Among the five peptide chains in the structures of TGF-β2,

Table 3 rms agreement among the cystines and the sequence identity between the growth factors[a]

	TGF-β2	PDGF-BB	β-NGF	hCG-α	hCG-β
TGF-β2		0.81	1.54	0.67	0.85
PDGF-BB	12		1.36	0.56	0.73
β-NGF	10	10		1.43	1.31
hCG-α	13	17	9		0.57
hCG-β	13	12	9	9	

[a] The upper diagonal shows the rms agreement in Ångstroms, calculated using all the atoms of the six knotted half-cystine residues, and the lower diagonal shows the number of identical residues based on the 3D structural alignment. All structural alignments are done with the program ALIGN (79).

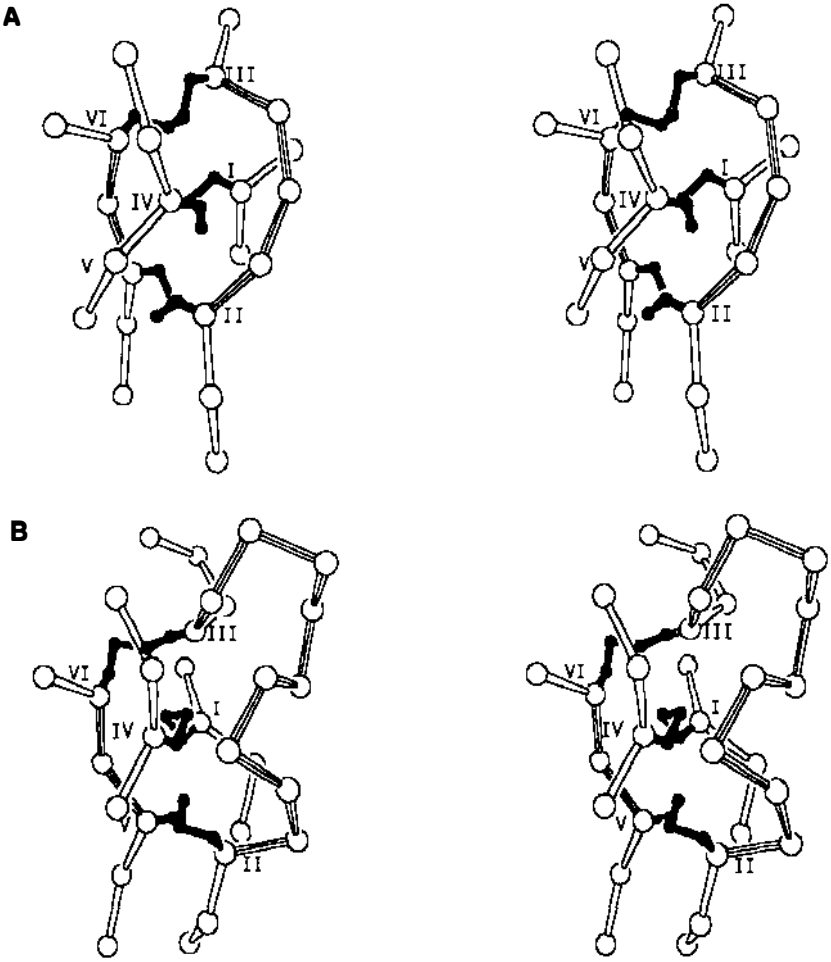

Figure 2 Stereo representation of the cystine knot. The six cystines participating in the three disulfide bonds are sequentially named CysI through CysVI. The disulfide pairings are between CysI and CysIV, CysII and CysV, and CysIII and VI. The topological knot is formed by passing the I-IV disulfide bond through the ring bounded by the II-V and III-VI disulfides and the connecting peptide backbones (only the C_α atoms are shown) in between (*shaded bonds*). (*A*) The eight-membered ring cystine knot, as observed in the structures of TGF-β2, PDGF, and hCG. (*B*) The 14-membered ring observed in the NGF cystine knot. ORTEP was used to prepare the figure (42). Although the C_αs of the cysteines of the proteins in the ring (*A*) superimpose on those in NGF (*B*), the conformations of their three disulfide bridges have the opposite chirality (67).

PDGF-BB, β-NGF, and hCG, four have an 8-membered cystine ring and one, β-NGF, has a 14-membered cystine ring (Figure 2*b*). Where only three residues lie between CysII and CysIII, as is the case for all members of the TGF-β and PDGF families and glycoprotein hormones,

the middle residue between the two cystines is always a Gly, i.e. a CXGXC pattern. The conservation of this Gly residue has a simple structural explanation, namely that any other amino acid in this position would imply severe steric hindrance with the backbone of CysI, whose position is fixed by means of disulfide bonding with CysIV. The requirement of a Gly residue is relaxed when the cystine ring becomes larger, as in the case of the NGF family. Furthermore, the rms differences among all the half cystines appear to correlate well with the size of the cystine-knot ring, as shown in Table 3. That is, the rms differences among TGF-β2, PDGF-BB, hCG-α, and hCG-β, which all have an eight-membered ring, and the CXGXC pattern are less than the rms differences between them and NGF, which has a 14-membered ring.

SIMILARITY IN β-STRANDS Apart from the cystine residues, the different families show little sequence similarity. Among TGF-β2, NGF, PDGF, and hCG, the sequence identities are well below 20% (see Table 3, lower diagonal). Nevertheless, the backbone conformations of these growth factors are remarkably similar, especially in the regions near the disulfide knot (Table 4). The results of Table 4, in which the backbone α-carbons have been superimposed, show that a large fraction of the C_α atoms can be paired to within 3.0 Å in most of the comparisons. Figure 3 illustrates the part of the backbone structure that can be superimposed among different growth factors, including a conserved twist in the middle of the fourth strand.

The conservation of the cystine-knot motif among these growth factors clearly indicates the importance of the disulfide bridges. They provide much of the fold's framework. Mutational analysis of TGF-β1 and PDGF (15, 31), in which the cystine residues are sequentially mutated throughout the sequence, showed loss of biological activity of the growth factor upon mutation of any of the six cysteine residues. In contrast, the cysteines that form intersubunit disulfide bonds are in

Table 4 Structure superposition among the alpha carbons[a]

	TGF-β2	PDGF-BB	β-NGF	hCG-α	hCG-β
TGF-β2		3.2	3.16	2.0	2.6
PDGF-BB	72		3.51	1.98	1.40
β-NGF	73	59		3.57	3.70
hCG-α	64	56	45		2.35
hCG-β	89	64	42	68	

[a] The upper diagonal shows the rms agreement in Ångstroms, and the lower diagnonal shows the number of alpha-carbon pairs used in the alignments.

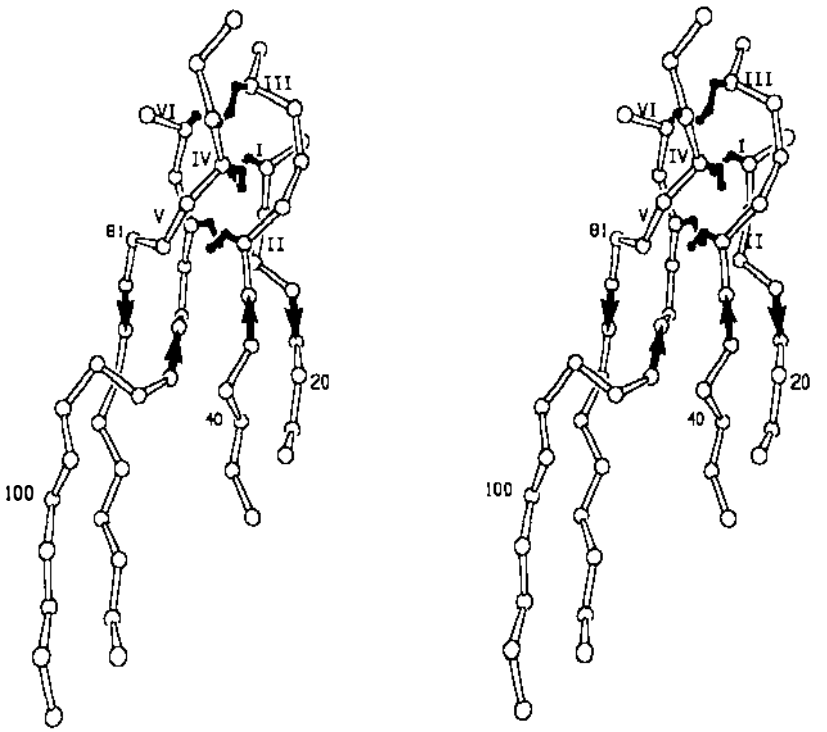

Figure 3 An α-carbon representation of the structurally conserved regions among TGF-β2, NGF, PDGF, and hCG. It includes the central cystine knot (*solid bonds*) and a four-stranded irregular antiparallel β-sheet (*open bonds*). A twist between the third and fourth strand is also observed in all four growth-factor structures. Arrows indicate the direction of the strands. ORTEP was used to prepare the figure.

some cases dispensable, e.g. the Cys43-Cys52 intersubunit disulfide bond of PDGF (44).

Apart from the disulfide knot and the conserved region of the four β-strands, the four growth factors share no structural resemblance. The N-terminal tail region and the regions between the conserved strands differ greatly. For example, in the region between the second and third conserved β-strands (or between CysIII and CysIV), all four monomers differ both in size and in conformation. PDGF-BB has the shortest connection in this region, only a six-residue β-hairpin loop. NGF has a longer and more complicated loop, an 11-residue loop, in between CysIII and CysIV. This region in both TGF-β2 and hCG has regular secondary structures. In TGF-β2, they are mostly helical, whereas both the hCG-α and hCG-β-subunits have a pair of long antiparallel β-strands. Sequence analysis reveals that these regions are also less con-

served within each individual family and thus may adopt different conformations even among the members of the same growth-factor family (24).

OTHER KNOTTED DISULFIDE STRUCTURES Besides the knotted disulfide structure assumed in these growth factors, other protein structures also have knotted disulfide arrangements. Examples are the potato carboxypeptidase A inhibitor (76), the scorpion neurotoxin, and the trypsin inhibitor EETI II (1, 20), which all have three knotted disulfides with the same pairing as the disulfides observed in the growth-factor structures, namely the I-IV, II-V, and III-VI cystine bondings. However, each forms its own unique 3D knot. Topologically, in all the growth-factor situations, the disulfide I-IV threads through a ring formed by II-V and III-VI. This threading disulfide is III-VI in the potato carboxypeptidase A inhibitor and the trypsin inhibitor EETI II structures, and II-V in the scorpion neurotoxin structure.

Comparison of the Dimer Structures

When the structures of NGF and TGF-β2 were published, a comparison revealed two interesting features (22). First, their protomer structures exhibit striking similarity, despite the lack of any sequence homology. Second, in contrast, their modes of dimer association differ profoundly. Since the publication of these reports, the structures of PDGF-BB and hCG have been added to the fold family, and each of these also has unique dimerization modes.

DIMER INTERFACE IS EXTENSIVE AND HYDROPHOBIC All members of the TGF-β, NGF, PDGF, and glycoprotein hormone families of growth factors form dimers, either homo- or heterodimers. Some dimers, such as the members of the TGF-β and PDGF families, are connected by one or two covalent disulfide linkages, and others, such as those of the NGF and glycoprotein hormone families, are connected by noncovalent, mostly van der Waals, interactions (Table 2). The amount of interface area buried in each dimer is very extensive, measuring 2600, 2340, 2200, and 4525 Å^2 (from 47) and 3860 Å^2 (from 97) for TGF-β2, NGF, PDGF-BB, and hCG, respectively. The excessively large interface area buried in an hCG dimer partly results from an additional separate interface formed between the so-called seat-belt loop of the β-subunit and the α-subunit. Most of the interface area in the structures of the four growth factors is hydrophobic in nature, which suggests that the main driving force for the formation of a stable dimer is the hydrophobic interaction.

STABILITY OF A MONOMER VS A DIMER Given the overall flatness and lack of a defined hydrophobic core of the protomer structure, we predict that a monomeric form of these growth factors is unlikely to assume a stable native conformation in solution. This prediction can be rationalized by comparing the area of the buried hydrophobic core of a monomer with that of a dimer. The calculated surface area shows that the monomers of TGF-β2, NGF, and PDGF bury approximately a total of 44, 43, and 33%, respectively, of their nonpolar surface area, whereas the dimers bury a total of 58, 57, and 54%, respectively. Miller et al surveyed the percentage of nonpolar surface area buried in 40 well-refined protein structures and found that protein molecules bury on average 55 $\pm$ 5% of their nonpolar surface area (64). Because the hydrophobic interaction, which is best measured by the amount of buried nonpolar surface, is the main stabilizing force of a protein structure, Miller et al's findings would argue that the three monomers lack sufficient hydrophobic stabilization to sustain their native conformations, whereas the dimeric forms bury a typical amount of nonpolar surface and thus are stable in solution. The monomeric forms of glycoprotein hormones may provide more buried nonpolar surface than those of TGF-β2, NGF, and PDGF because they are heavily glycosylated upon expression and thus may exist as stable monomeric forms in solution.

THE UNIQUENESS OF THE DIMERIZATION MODES The dimers whose 3D structures have been characterized so far all differ both in the orientations of their respective monomers and in the locations of their dimer interfaces. One (NGF) dimerizes in a head-to-head orientation, and the other three, TGF-β2, PDGF-BB, and hCG, dimerize in head-to-tail orientations (Figure 4). In terms of the locations of their interfaces (Figure 5, color plate), NGF uses the relatively uncurled face of the four-stranded β-sheet to form the contact surface. TGF-β2, in contrast to NGF, adopts the opposite side of the β-sheet, the curled up face, as its dimer interface. PDGF-BB, on the other hand, dimerizes on the side of the β-sheet so that most of the interface area is mediated by the first two strands of the β-sheet and its N-terminal tail. Finally, the dimer interface of human chorionic gonadotropin appears to be located partly on the curled up face of the β-sheet and partly on the side of the β-sheet's first strand, resulting in an overlap of the contact area with that of TGF-β2 and PDGF-BB. These very different dimer formations result in very different locations for the hydrophobic residues in their respective protomer structures. Consequently, all the existing 3D-1D profile–based, sequence-alignment algorithms fail to show any similarity between the monomers. This makes predicting a member of this superfamily fold from the sequence alone particularly challenging.

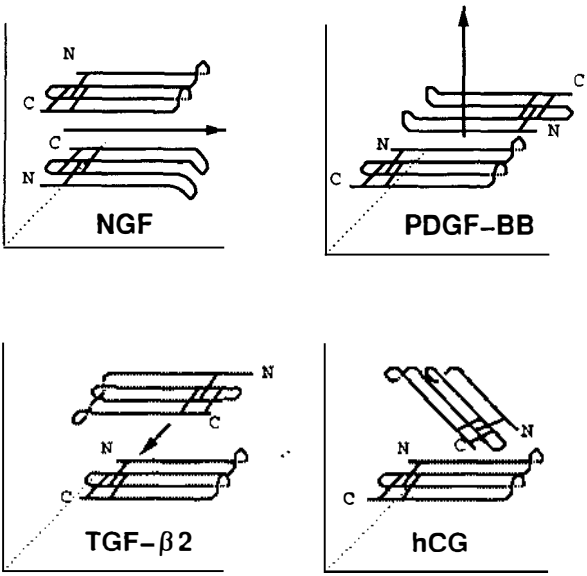

Figure 4 Schematic representation of the different dimer forms. Each protomer is represented by a set of three knotted disulfide bonds and a four-stranded antiparallel β-sheet. N and C label the N and C termini. The molecular two-fold axis that relates two monomers is shown with an arrow in each case except hCG, in which there is only a pseudo two-fold axis between the α- and β-subunits.

Finally, most of these growth factors, with the possible exception of glycoprotein hormones, are expressed first as latent forms with propeptides similar to or larger than the mature growth factors themselves (63, 83). The functions of these propeptides remain largely unknown, although they are believed to take part in the activation regulatory processes controlled by the cell surface proteases (65, 92). Given the fact that the monomers lack well-defined hydrophobic cores, protein folding and dimerization become a highly cooperative process. Therefore, propeptides may also function as chaperones to facilitate the folding of the mature peptides.

RECEPTORS AND RECEPTOR BINDING

The structural similarity among these growth factors does not extend to either the extracellular ligand-binding domain or the intercellular kinase domain.

Extracellular Binding Domain

No sequence homology has been observed in the ligand-binding domain of these growth-factor receptors, and no 3D structure for them has been determined. Three major forms of TGF-β receptors, types I, II, and III TGFβR, have been cloned (100–103). Types I and II, which have intracellular kinase domains, are the signal-transducing receptors. The major ligand-binding receptor, type II TGFβR, has a relatively small ligand-binding domain of 136 amino acids that contains cysteine-rich segments. These receptors exhibit no recognizable sequence homologies with other receptor families.

NGF has two forms of receptor, the low-affinity (LNGFR, $p75^{NGFR}$) and the high-affinity form (HNGFR, $p140^{trkA}$). The extracellular domain of $p75^{NGFR}$, which can bind to NGF, BDNF, NT-3, and NT-4, has four cysteine-rich regions similar to those in TNF receptors, and the extracellular part of the high-affinity receptor $P140^{trkA}$, a member of the *trk* protooncogene family, has two cysteine-rich domains and two immunoglobulin domains.

The extracellular portion of the PDGF receptor consists of 5 immunoglobulin-like domains, whereas the extracellular part of hCG receptor contains 14 leucine-rich repeats (LRRs) (61). The sequence of the ribonuclease inhibitor has a similar number of LRRs (97); the structure of these receptors assumes a horseshoe shape.

Receptor Dimerization

Ligand-induced receptor dimerization has been observed crystallographically in two growth factor–receptor systems. One is the human growth hormone system, in which one molecule of hGH binds to two receptor molecules asymmetrically and thus facilitates the receptor dimerization (26). The other example is the recent solution of the complex structure between TNF and TNF receptor. In this case, TNF itself exists as a trimer, and it binds symmetrically to a receptor trimer (5). Because all the cystine-knot growth factors are dimers, the signal-transduction mechanism may involve the growth factor–induced receptor dimerization. In fact, in the inhibition of cell growth signaled by TGF-β, evidence indicates that the binding of TGF-β1 to its cell surface type II receptor—the high-affinity receptor—causes the receptor to dimerize with the type I TGF-β receptor—the low-affinity receptor—and it is this receptor complex that leads to the subsequent kinase activation (9, 96). The PDGF and NGF receptor systems represent similar cases (12, 21, 38, 62).

The location of most of the proposed receptor-binding sites remain

largely speculative because of insufficient mutagenic analysis and the absence of a ligand-receptor complex structure. These proposed sites reside in the loop regions between the four structurally conserved β-strands and are outside the cystine-knot motif. Because these growth-factor regions differ greatly at both the sequence and structure level, different families of growth factors probably recognize their receptors in entirely different manners.

FUTURE PERSPECTIVES

The situation in which the promoter sequences of all four growth factors share extensive structural resemblance and yet lack any significant sequence homology represents an extreme example of divergent evolution. The sequence divergence is nearly complete, except for the six knotted cystine residues. However, the 3D fold is preserved throughout evolution. By which process did these growth factors evolve? What was the earliest form, a dimer or a monomer? Another interesting issue is that all the members of this cystine-knot superfamily exist primarily in dimeric forms. Are there other forms apart from the four known dimers? Furthermore, many dimers can form both homo- and heterodimers that have different biological activities. For example, the activins and inhibins, members of the TGF-β family, are formed by differential dimerization of three polypeptide chains, α, βA, and βB. When the α-chain pairs with βA or βB, it produces either inhibin A or inhibin B, which inhibit the production of pituitary FSH, gonadal sex steroids, and placental hormones (98). However, when βA forms a homodimer or dimerizes with βB, two activin molecules result that have an effect on growth regulation opposite to that of the inhibins (74). Whether this intrinsic property of differential dimerization is part of the cell regulatory machinery remains unknown. In some situations, such as PDGF, the isoform population does appear to be regulated (37). Finally, a very challenging question is how to predict, based on sequence information, other members of this superfamily fold. How many more growth factors are there that assume the same fold? The answer to this question requires a better understanding of the fold, especially the determinants of the fold other than the cystine residues. Our current knowledge based on the four growth factor structures has not yet provided a tool to accomplish this goal.

ACKNOWLEDGMENTS

We thank Dr. NW Isaacs for making hCG coordinates available to us, Dr. GH Cohen for discussions related to the structural alignment, and

Drs. A Roberts and S-W Chen for their helpful comments on the manuscript.

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