1993

Cytokine structural taxonomy and mechanisms of receptor engagement

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Seven discrete families of cytokines have been identified by sequence and structural analysis. Within this diverse set of protein folds, the hematopoietic growth factors and tumor necrosis factor-like cytokines display a remarkable degree of variation upon their respective topological frameworks. In addition, prototype X-ray structures of ligand–receptor complexes reveal two different strategies of receptor engagement.

Current Opinion in Structural Biology 1993, 3:815–827

Introduction

Cytokines and growth factors serve as signal carriers in a dynamic cellular communications network. These pleiotropic mediators act synergistically or antagonistically to orchestrate the behavior, proliferation and death of cells, acting directly and/or by regulating the expression of other cytokines. All cytokines bind to the extracellular domains of transmembrane cellular receptors and trigger intracellular responses mediated, in what appears to be a universal paradigm, by ligand-induced aggregation of the receptors themselves [1•].

Cytokines are a diverse group of proteins. Many of these molecules and their cognate receptors were functionally and genetically characterized during the 1980s [2]. In recent years, with the recognition of their potential pharmacological value, commercial biotechnology firms have developed large-scale expression systems for a variety of cytokines, for which structural biologists have generated a wealth of structural detail. With the diversity of cytokine sequences, it seemed at first that each new structure would reveal a novel and perhaps unique protein topology. Instead, cytokine structures have been found to cluster into a few distinctive protein folds (Table 1) [3], a striking reminder of global, simplifying themes in protein architecture. It is also notable that the cellular receptors for these cytokine families have been found to share a small number of binding motifs [4•]. With the consolidation of cytokine and receptor structural families, the molecular details of a particular receptor–ligand complex have general implications for a broad set of interacting molecules.

The principal thrust of cytokine structural biology is to determine how receptors specifically recognize and respond to their ligands. The remarkable primary sequence diversity within families of cytokines and their receptors indicates that complementary binding surfaces have co-evolved to generate rapid functional divergence of ligand receptors. In the present work, we review structural studies of the hematopoietic cytokines and tumor necrosis factor (TNF)-like molecules, and of two corresponding, prototype receptor–ligand complexes that have begun to address some of these questions. We focus on the evolutionary plasticity revealed in primary sequence and tertiary structural variation within these structural superfamilies, and consider how the two unrelated protein motifs are adapted to alternative strategies of receptor engagement.

The hematopoietic growth factor family

All members of the hematopoietic growth factor family share a common four-helical bundle topology (Fig. 1) first observed in the structure of porcine growth hormone (GH) [5]. The first and second pairs of helices in this fold are linked by long overhand connections packed against one side of the molecule. This unique topological feature ensures that the arrangement of helices in the bundle core resembles that of a classic antiparallel four-helix bundle in spite of the fact that the chain-sequential helices A and B, and C and D are parallel [6]. In this manner, the GH-like helical cytokine fold can best be described as a two-layer packing of antiparallel helix pairs A–D and B–C with a 'skew' angle Ω, AD–BC describing the varying angle

Abbreviations

CRD—cysteine-rich domain; **G-CSF**—granulocyte colony-stimulating factor; **GH**—growth hormone; **GM-CSF**—granulocyte/macrophage colony-stimulating factor; **IL**—interleukin; **INF**—interferon; **Lc**—long-chain; **M-CSF**—macrophage-colony-stimulating factor; **rmsd**—root mean square deviation; **Sc**—short-chain; **TNF**—tumor necrosis factor; **TNFr**—tumor necrosis factor receptor.

 ISSN 0959-440X

Table 1. Cytokine structural superfamilies.

Structural class	Protein fold	Molecule	Homology	Receptor type	Reference
(β/β)	Cystine knot				[3]
		NGF	X	Ig	[51]
		TGF-β	X	Cys-rich	[52,53]
		PDGF B	X	Ig	[54]
(β/β)	β-Trefoil				[55]
		IL-1α	X/N	Ig	[56]
		IL-1β	X	Ig	[57–59]
		IL-1 ra	N	Ig	[60]
		aFGF	X	Ig	[61]
		bFGF	X	Ig	[61–64]
(β/β)	β-Jellyroll				[36]
		TNF-α	X	TNFR Cys-repeat	[34,35]
		LT	X	TNFR Cys-repeat	[37,39••]
		CD40-L	H	TNFR Cys-repeat	[27]
(β/β)	S-S rich β-meander				[65]
		EGF	N	Cys-rich?	[65,66]
		TGF-α	N	Cys-rich?	[65,67]
(α + β)	Open-face β-sandwich				[68]
		PF-4	X	Serpentine	[69]
		IL-8	X/N	Serpentine	[70,71]
		MCP-1	H	Serpentine	[72]
(α + β)	Small, S-S rich α/β				[73]
		Insulin	X/N	Cys-rich?	[74,75]
		IGF-1	N	Cys-rich?	[76,77]
		Relaxin	X/H	?	[78,79]
(α + α)	4-α-Bundle				[6]
		GH	X	CD4D2	[5,14•]
		G-CSF	X/N	CD4D2	[80,81]
		IFN-β	X	CD4D2	[82]
		IFN-α	H	CD4D2	[83]
		IFN-γ	X	CD4D2	[84•,85]
		IL-2	X/N	CD4D2	[86,87]
		GM-CSF	X	CD4D2	[7,88]
		IL-4	X/N/H	CD4D2	[89–93]
		IL-5	X	CD4D2	[17•]
		M-CSF	X	Ig	[16•]

Molecule: CD40-L, ligand of the CD40 antigen; CSF, colony-stimulating factor; G-CSF, granulocyte-CSF; GM-CSF, granulocyte-macrophage-CSF; M-CSF, macrophage-CSF; EGF, epidermal growth factor; FGF, fibroblast growth factor; GH, growth hormone; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; LT, lymphotoxin; MCP, monocyte chemo-attractive protein; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PF, platelet factor; TGF, tissue growth factor; TNF, tumor necrosis factor. Homology; the structural family to which the cytokine belongs as determined by X-ray crystallography (X), nuclear magnetic resonance spectroscopy (N) or nomology modelling based on primary sequence alignments with other members of the family(H). Receptor: CD4D2, cell-surface glycoprotein domain-2-like fold; Ig, immunoglobulin-like fold; TNFR, TNF receptor.

between the helical faces of the molecule. For comparative purposes, the topology is a helical version of the 'Greek key' topology found in β-β class proteins [7]. Instead of the right-handed 'swirl' characteristic of β keys, however, the helix-helix progression is left-handed. Accordingly, Presnell and Cohen [6] label the GH bundle fold as a left-handed type 2 four-helix bundle.

There is little discernible primary sequence identity among hematopoietic cytokines [2]. A structural re-

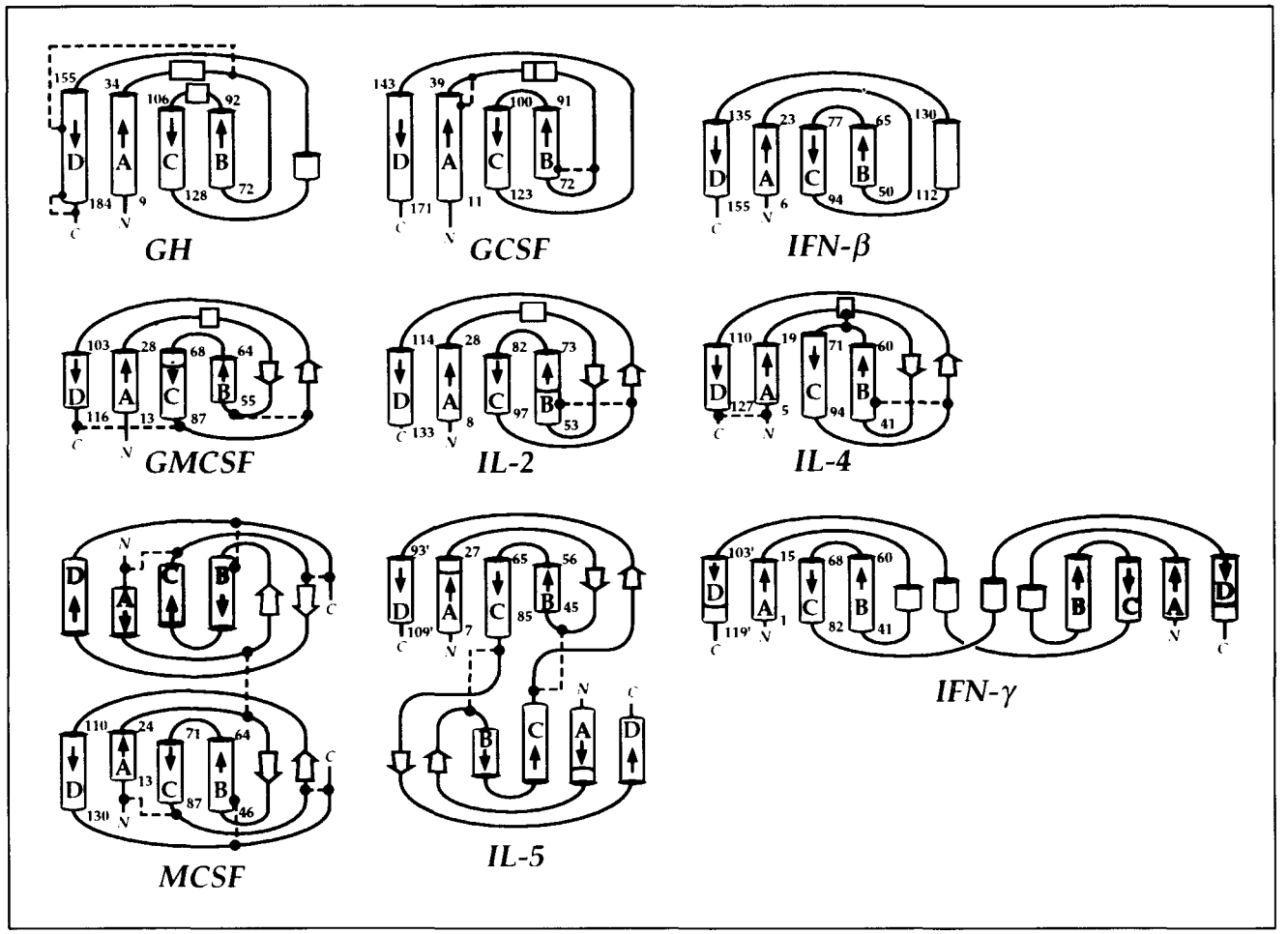

Fig. 1. Comparative anatomy of helical cytokine folds. All folds are shown in the left-handed 'Greek key' representation, adapted from the work of Hill *et al.* [16•]. The four helices of the core bundle are labeled A–D; only the endpoints of these latter helices (and not secondary structure present in loops) are marked, for clarity. Dashed lines depict cystine crosslinks. The molecules are roughly grouped by structural subtype; the top tier of GH, G-CSF and IFN-β cytokines fall into the Lc category whereas the remaining GM-CSF, IL-2, IL-4, IL-5, M-CSF and IFN-γ are described as Sc cytokines. Distinct dimer configurations are evident for IL-5, M-CSF and IFN-γ; reverse-shaded features in M-CSF or IFN-γ subunits represent the 'back' faces of a symmetry-related partner (Fig. 3).

lationship was first suggested by similar patterns of predicted helix propensity and amphipathicity; a parallel genetic homology was indicated by the mapping of these protein features to common exons in cognate cytokine genes (Figs 1–3) [8,9]. In a series of papers, Bazan [8–11] and others [12] have extended the helical cytokine family to include not only the endocrine hormones GH and prolactin, but also a number of interleukins, colony-stimulating factors, neuropoietic cytokines and interferons. Many of the predictions formulated in those reports have been fulfilled in a recent cascade of structure determinations, both by X-ray crystallography and multi-dimensional heteronuclear NMR. At this time, the three-dimensional folds of nine members of the hematopoietic growth factor family have been described (Table 1). The structures of others have been inferred by homology, by predictive model building or mutagenic experiments coupled to spectroscopic measurements [13].

Variations on a motif

The occurrence of similar folds in apparently unrelated protein chains is a reminder of the economy of nature, and a challenge to protein-folding theorists. Cytokines serve as proteinaceous 'information carriers' and most of the folded structure acts as a scaffold for a surface that is 'read' by a specific receptor. In the case of the helical cytokines, this functional imperative involves the evolutionary preservation of a specific packing geometry between the A and D helices of the bundle core that form a major part of the receptor contact site; other core helices suffer compensating shifts. Sequence variation upon the conserved backbone helps generate a unique topographic surface for a specific receptor–ligand pairing [14•].

Structural comparison of the nine available helical cytokine folds reveals a fundamental division of the topologically related molecules into two divergent

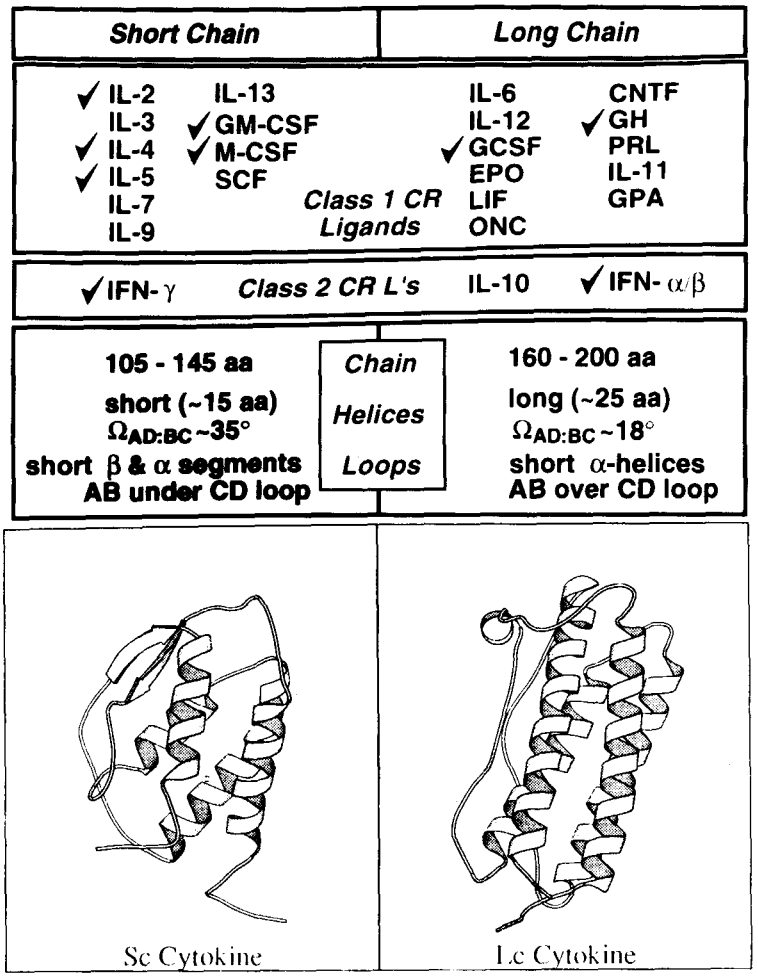

Fig. 2. Helical cytokines are classified into Lc and Sc groups. Representative Lc and Sc folds (G-CSF and GM-CSF, respectively) in MOLSCRIPT rendering [94] show distinctive structural features that persist in a similar topological framework. The inset box defines chain, helix and loop differences derived by structural comparison of available cytokine folds (Table 1); in accord with these points, and aided by predictive methods [9], the existing pool of hematopoietic cytokines has been divided into Lc and Sc ligands of class 1 and 2 hematopoietic receptors [95]. Tick marks distinguish cytokines of determined structure (Table 1).

subgroups, designated here as the short-chain (Sc) and long-chain (Lc) cytokines (Fig. 2). This structural taxonomy is based on several simple criteria (aside from chain length) that describe helix length and packing modes, and features of the long crossover loops (Fig. 2). The Sc cytokine group, founded by the closely similar folds of granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-2, IL-4 and macrophage-colony-stimulating factor (M-CSF) [Table 1], comprises molecules of ~105–140 amino acids in length, helices of ~15 amino acids in length, typically large packing angles between the A–D and B–C helix layers and long crossover loops that contain two short and twisted antiparallel β-strands. In addition, the AB crossover loop arches over the helix bundle (often folded in a short helical stretch) and packs behind the antiparallel CD loop chain (Fig. 2). The general appearance of an Sc cytokine is that of an oblate ellipsoid. A disulphide bridge often tethers helix B to the passing CD loop chain (Fig. 1). Other hematopoietic cytokines that are strongly predicted to adopt this characteristic fold include IL-3, IL-7, IL-9, IL-13 and stem cell factor.

The Lc cytokine group includes three cytokine structures: the prototype GH, granulocyte colony-stimulating factor (G-CSF) and interferon (IFN)-β (Table 1). These molecules contain helices (~25 amino acids) that are more tightly packed in structures that approximate elongated cylinders. The loops lack the β-sheet structure of the Sc cytokines, and produce a distinct packing of the AB loop over the top of the D helix and appended CD loop. All three Lc structures also feature non-equivalent extra-core helices in the long crossover loops; in particular, IFN-β has a long helical segment in the CD loop that packs tightly against the four-bundle core. These loop helices may be capricious indicators of cytokine relatedness; for example, the (receptor-bound) human and (soluble) porcine homologs of GH differ significantly in the secondary structure of AB and CD loop excursions, a fact that may reflect conformational changes upon receptor contact [14•]. Other cytokines that closely resemble GH and G-CSF include prolactin, IL-6, IL-11, IL-12, erythropoietin, leukemia inhibiting factor, oncostatin M and ciliary neurotrophic factor; in turn, the shorter helix bundle of IFN-β (Fig. 1) should recur in the folds of IFNα and IL-10.

Least-squares superposition of Sc or Lc cytokine frameworks shows a good correspondence between the four-component helices of the bundle core and certain stretches of the loop regions, such as the twisted β-sheet segments of Sc cytokines (Fig. 3); typically, 60–70 C_α pairs match with a root mean square deviation (rmsd) of 2–2.7 Å between Sc molecules whereas Lc GH superimposes 97 (76) C_α positions with G-CSF

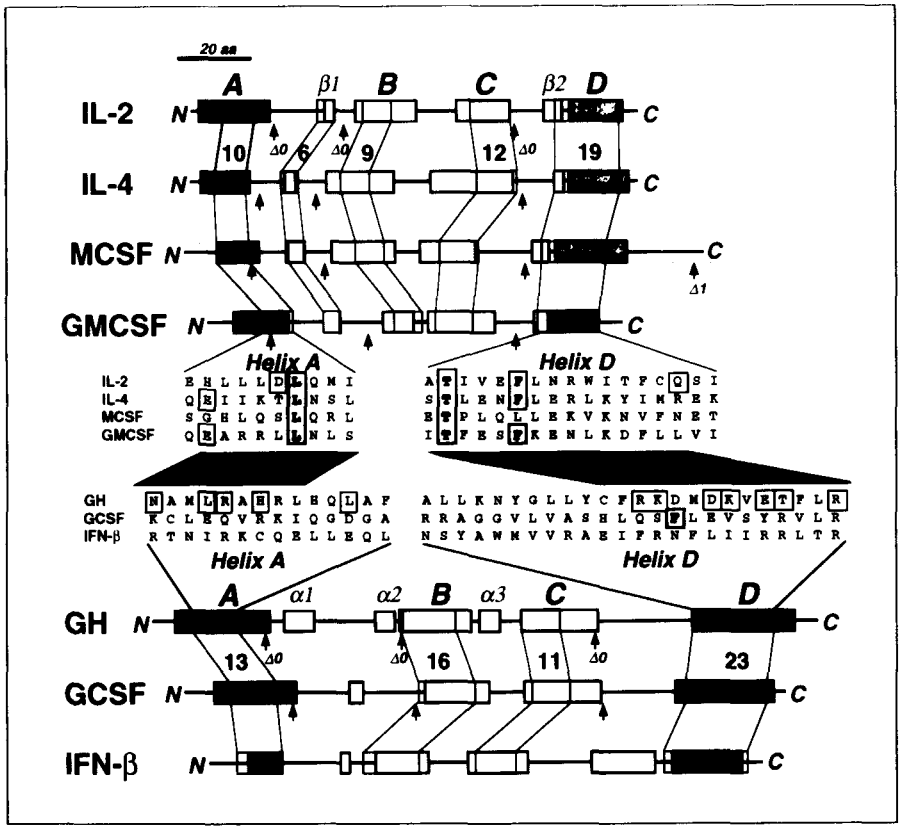

Fig. 3. The superposition of available three-dimensional frameworks produces a structural correspondence of chains essential to an accurate alignment of helical cytokine sequences. These matches in general (for both the Sc and Lc cytokine folds) comprise the four core helices; in addition, Sc cytokines feature overlapping strands β1–2 in the long crossover loops. Multiple alignments of Sc and Lc sequences in the A and D helix regions are shown. Only two residues are completely conserved among the Sc cytokines (boxed and shaded); in addition, an aromatic residue is frequently observed in the D helices of both Sc and Lc cytokines (including predicted structures) that packs into the interface between B and D helices and the crossover loops. Residues implicated in GH-receptor binding are boxed; by structural homology, the equivalent residues of related cytokines can be identified.

(IFN-β) to better than 2.6 (1.6) Å rmsd. Structurally conserved regions for the Sc and Lc cytokines, respectively (Fig. 3), then serve as a starting point for the global comparison of Sc and Lc chains. Typical pairwise 'scores' include M-CSF versus GH with 67 C_α pairs at 1.73 Å rmsd, GM-CSF versus G-CSF with 56 C_α pairs at 2 Å rmsd and IL-4 versus IFN-β with 60 C_α positions at 2.4 Å rmsd. A summary of this 'all-against-all' superposition is provided in Fig. 3 with the structural correspondence between the helix A and D segments that are implicated in receptor binding for GH [14•]. If the overlay between Sc and/or Lc cytokines is restricted to the A–D helix pairs, the fit is considerably better, with 30–40 C_α positions superimposing to 1.2–1.5 Å rmsd.

It is striking that only two amino acids are completely conserved among the four available Sc structures; not one conserved residue is evident among the Lc chains (Fig. 3). But if the sequences of other hematopoietic cytokines (Fig. 2) are included in a structurally accurate alignment, other patterns of conservation become evident. Notably, the Sc cytokines feature an exposed acidic residue in the center of the helix A receptor-binding region (the analogous residue in IL-2 is displaced by one helical turn; Fig. 3), as predicted earlier [15]; the Lc cytokines frequently have a spatially equivalent basic residue in helix A. Other regions of the cytokine fold do not appear to have any conserved, exposed residues. The amino-terminal part of helix D, however, contains a buried tyrosine or phenylalanine aromatic group that is quite well conserved among both Sc and Lc cytokines (Fig. 3). The structural and functional similarity of Sc and Lc cytokines provides a strong argument for a homologous relationship rather than convergence to a favorable fold. A parallel genetic homology is observed between Sc and Lc cytokine genes that have a common intron-exon structure [8]; accordingly, intron positions mapped to the respective protein chains are located in similar structural contexts (Fig. 3). Genetic acceleration/deletion of sequences in exon boundaries that map to helix ends and exposed loop regions of the encoded cytokines could serve to elongate/shorten helices and loops; this evolutionary mechanism may explain the divergence of Sc and Lc cytokines.

IFN-γ has characteristics of both Sc and Lc cytokines. Each IFN-γ subunit requires ~120 residues to complete the bundle fold (like an Sc cytokine) but has short helices in the crossover loops that feature an Lc-like AB-over-CD crossover (Fig. 1). This classification is best resolved by a structural superimposition of the IFN-γ interdigitated subunit framework with both Sc and Lc folds, which shows that the skewed helix bundle of IFN-γ is more similar to that of Sc cytokines.

Most of the hematopoietic cytokines function as monomers. Within the Sc family of cytokines, however, three distinct modes of dimer association are observed in the folds of IFN-γ, M-CSF and IL-5. The twofold axis of symmetry that relates subunits of IFN-γ runs approximately parallel to the axes of the monomer helix bundles, orienting the subunits in a side-by-side configuration with interpenetrating chains; the CD loop and D helix of each subunit originate from the dyad-related partner (Fig. 4). Monomers of the M-CSF dimer

are related by a twofold axis that runs perpendicular to the helix bundle axes. The subunits are associated head-to-head (opposite the adjacent amino and carboxyl termini) with the dyad-related AB and CD loops forming a subunit interface that buries 850 Å^2 of surface area [16•]. Whereas M-CSF molecules are linked by a disulfide bridge between symmetry-related Cys31s in the packed AB loops that lie on the dyad axis, the structurally related stem cell factor forms a non-covalent dimer that lacks a cysteine at the corresponding position [11]. IL-5 dimers differ from M-CSF in that the helix bundles are aligned 'tail-to-tail'; like IFN-γ, monomers of IL-5 interpenetrate extensively, burying ~7000 Å^2 of subunit surface area [17•]. As a result, the CD loop and D helix in each bundle is contributed by the opposite subunit (Fig. 4). Although the two dimers bear a superficial resemblance, the putative receptor A and D helix surfaces of adjacent subunits are displayed on the same face of the IL-5 dimer, but on opposite sides of the M-CSF dimer.

Receptor binding and specificity

The receptors for hematopoietic growth factors belong to a large family of structurally related molecules comprising two or more immunoglobulin-like modules. GM-CSF, G-CSF, IL-2 to -7, GH, prolactin and erythropoietin engage type I extracellular domains composed of two repeats similar in structure to that of CD4 T-cell receptor. IFN-α, -β and -γ recognize members of the closely related type II family. G-CSF and stem cell factor engage yet a third member of the immunoglobulin-like receptor family which, unlike the former two, is linked to an intracellular tyrosine kinase domain. Except for recurrent amino acid patterns indicative of a common architecture, there is little sequence identity among this disparate group of receptors, suggesting that a correspondingly large variation can be expected in the receptor-binding surfaces of their ligands. Indeed, although all hematopoietic factors appear to recognize immunoglobulin-like receptor domains, the reverse is not the case. Such structurally unrelated cytokines as IL-1β (a β-trefoil fold) and platelet-derived growth factor (a cysteine knot factor) engage receptors of the type III family. Thus, cytokine-binding surfaces complementary to the receptor domain can be achieved within a variety of structural contexts.

Hematopoietic factor receptors are activated by ligand-dependent formation of either homo- or heterodimeric receptor molecules [1•]. Ligands, if they act as monomers, must present distinct surfaces to each receptor, or if dimeric, may be expected to present identical, symmetry-related binding surfaces (Fig. 4). Growth factor, which has been characterized by high-resolution X-ray crystallography as a complex with its receptor, clearly falls into the former category [14•]. In contrast, IL-5 [18], M-CSF [16•] and IFN-γ [19] bind as dimers to a single (presumably homodimeric) receptor. Both the structural results and those undertaken by mutagenic studies of GH and prolactin indicate that the two surfaces are not energetically equivalent. Formation of the (receptor)$_2$·GH complex is a sequential reaction, involving, first, the binding of a high-affinity GH site to one receptor molecule, followed by attachment of a second low-affinity binding GH site to the second receptor molecule [20•]. This binding mechanism has been exploited in the design of GH variants that act as competitive inhibitors of receptor aggregation by virtue of mutations localized to the low-affinity site.

The high-affinity site in GH, comprising 1230 Å^2, consists of the amino terminus of helix A, the mid-section of the AB loop and the carboxy-terminal half of the D helix. The less extensive (900 Å^2) low-affinity site encompasses the amino terminus of the protein and a few residues in the middle of helix C. Mutagenic analysis suggests that the prolactin receptor, which binds GH with Zn^{2+} as a cofactor [21], seeks a high-affinity site which is shifted, relative to the GH receptor-binding site, toward the carboxyl terminus of helix A (part of the Zn^{2+}-binding site) and the amino terminus of helix D, a region further along the bundle axis [22•]. The differences in site selection by two different receptors for the same hormone suggest that binding surfaces for the hematopoietic growth factors may diverge rapidly in evolution. The structures of other cytokine–receptor complexes in this family are not yet available; however, studies of point-deletion and chimeric mutants, notably of GM-CSF [23], suggest that homologous surfaces are involved in receptor binding by other hematopoietic cytokines. The dimerization surfaces of IFN-γ, M-CSF and IL-5 do not occlude this canonical binding site, but it is apparent that the each of the three cytokines present themselves to their receptors in different orientations (Fig. 4).

The TNF family

TNF-α (also known as cachectin) and its T-cell derived relative lymphotoxin (TNF-β) have long been associated with a complex array of inflammatory, immunomodulatory, antiviral and cytotoxic activities [24]. All of the members of the TNF family except TNF-β are type II membrane proteins. The cell-surface bound form of TNF-α, for example, is anchored to the plasma membrane by an amino-terminal hydrophobic anchor sequence [25], which can be cleaved to yield a soluble trimer. The TNF family includes several ligands expressed primarily by lymphoid cells. The ligand for the CD40 receptor (CD40L) exhibits 25.7% sequence identity to TNF-α, and 24.3% to TNF-β. This ligand is expressed as a surface protein by $CD4^+$ T lymphocytes and binds a TNF receptor-like receptor presented on B cells, initiating B-cell development and proliferation [26]. Unlike TNF-α, no soluble forms of the CD40 receptor ligand are produced. By computer-aided modeling energy minimization and one to three-dimensional profile analysis, the sequence of this ligand has been shown to be compatible with the tertiary and quater-

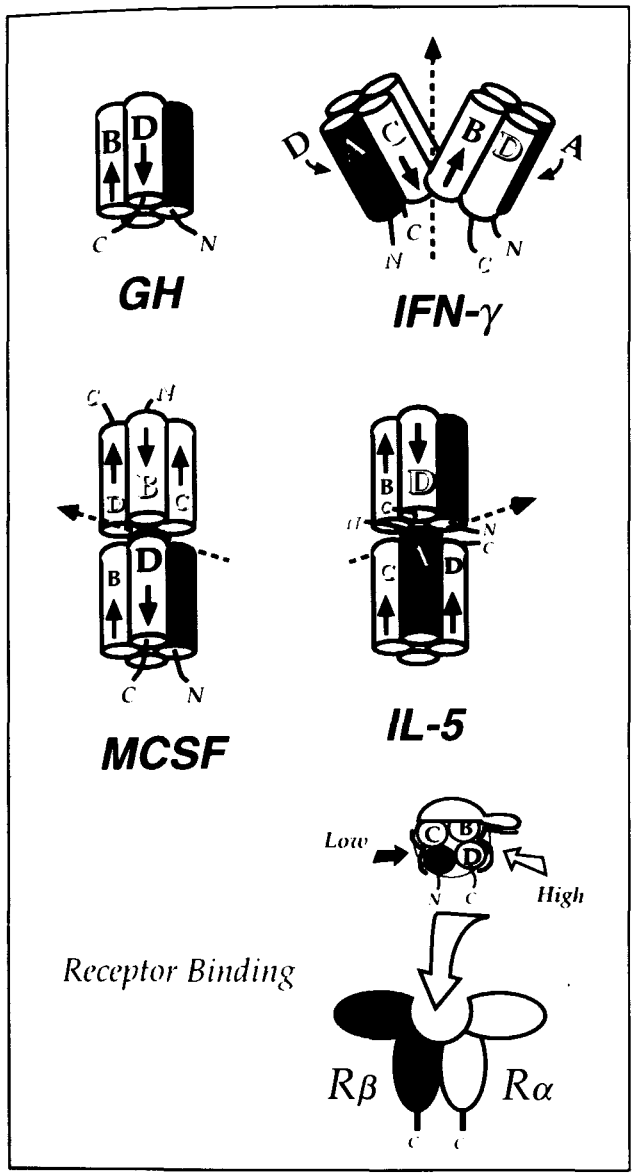

Fig. 4. Observed dimerization modes of helical cytokines include representative single-subunit cytokine bundle (labeled GH) with a simplified cluster of four antiparallel core helices labeled A–D (helix directions indicated by arrows) omitting connecting loops. The IFN-γ, IL-5 and M-CSF dimers show distinct dimer types. Whereas M-CSF is a 'head-to-head' disulfide-linked dimer of Sc subunits, IL-5 and M-CSF show a more complex interweaving of helices that results in the topological sharing of CD loop and D helix between adjacent subunits; these are distinguished by helix labels in outline notation. Even then, IFN-γ appear as a 'side-by-side' bundle dimer whereas IL-5 is a 'tail-to-tail' dimer. The inset box provides an end-on view (corresponding to the end of bundle where amino and carboxyl termini converge) of the prototype interaction between a GH-type helical cytokine bundle and a receptor complex formed by Rα and Rβ subunits. The 'cap' that now tops the cytokine bundle is meant to describe the appearance of the long crossover loops that pack against the helix core; in particular, these loops invariably form a loose structure alongside helix D that is akin to the 'brim' of the cap structure. The large and small arrows pointing to concavities between the D helix and the loop brim, and between the A and C helices, respectively denote the high- and low-affinity binding epitopes of the cytokine. As observed with the GH receptor–ligand complex, the deep high-affinity site is recognized by the Rα subunit whereas the shallow low-affinity site forms the Rβ-binding site.

nary framework of TNF-α and TNF-β [27]. The ligand for the CD27 receptor also belongs to the TNF family, having 19–24% sequence identity with other family members [28].

The two receptors that recognize TNF-α and TNF-β are members of the low-affinity nerve growth factor receptor superfamily [29]. The extracellular domains of these molecules contain three to four copies of a 40-amino-acid quasi-repeat distinguished by a characteristic pattern of six cysteine residues (cysteine-rich domains or CRDs). In spite of their ability to engage both TNF-α and TNF-β, the two receptors (one called p55, type I or TNFr-1; the other p75, type II or TNFr2) have few other features in common. Several other receptors, including the Fas antigen implicated in thymocyte apoptosis [30] and a variety of other antigens expressed on the surface of lymphoid cells [31•] including CD40 (B cells), CD30 (a lymphoid activator), OX40 (T cells), 4-IBB (murine T cells), CD27 (thymocytes and activated T cells) belong to the TNF receptor superfamily. The extracellular domains of the TNFrs are linked through a single transmembrane segment to a cytoplasmic domain. Though approximately the same size, the cytoplasmic domains of TNFr1 and TNFr2 show little homology to each other or to cytoplasmic signaling domains of other receptors. Evidence is mounting that the two TNFrs may be linked to different signal-transduction pathways [32,33]. Bazan [31•] has suggested that TNFrs fall into three distinct families, of which TNFr1 and TNFr2 represent two.

Jellyroll trimers

Independent investigations of TNF-α crystallized under two different conditions [34,35] have revealed a homotrimer of subunits intimately arranged around a threefold axis of symmetry (Fig. 5). The fold of the TNF subunit is virtually identical to that of icosahedral virus coat proteins, in particular, the economical structures of simple RNA plant viruses [36]. The primary sequence of TNF bears no detectable identity to the virus proteins. Lymphotoxin, or TNF-β, which is 33% identical in primary sequence, has essentially the same tertiary and quaternary structure as TNF-α [37]. The subunits are arranged in a cone-shaped trimer, with the adjacent amino and carboxyl termini of each subunit extending from the broad base. Subunits are packed so that the edge of the β sandwich of one subunit abuts the inner face of the neighboring sheet. In both structures, trimers are organized about an intermolecular hydrophobic core. In TNF-α, the core comprises mostly β-branched hydrophobic side chains, whereas in TNF-β, the core residues are mostly aromatic. Strands designated (in the nomenclature adopted by Eck *et al.* [37]) A″, A, H, C and F, in order of adjacency, form the inner sheet of the sandwich whereas the sheet comprising B′, B, G, D and E is exposed to solvent. (In the convention used by Chelvanayagam *et al.* [36],

the strand designations for the same two jellyroll sheets are B″, B, I, D, G and C′, C, H, E, F, respectively; Fig. 5.) The A′,A″,B′ assembly is really an extension of the loop that connects the first two strands of the canonical jellyroll fold. Intersubunit contacts are mediated by residues in strands E, F, C, H and A. The eight residues identically conserved among all members of the TNF family are involved in intrasubunit packing contacts. Interactions that hold trimers together are thus not universally conserved within the family. Residues involved in receptor recognition reside mostly in the strands and loops that line the subunit interface, A-A″ and D-E. Surprisingly, TNF-α and TNF-β have few surface features in common, despite their ability to bind the same receptors.

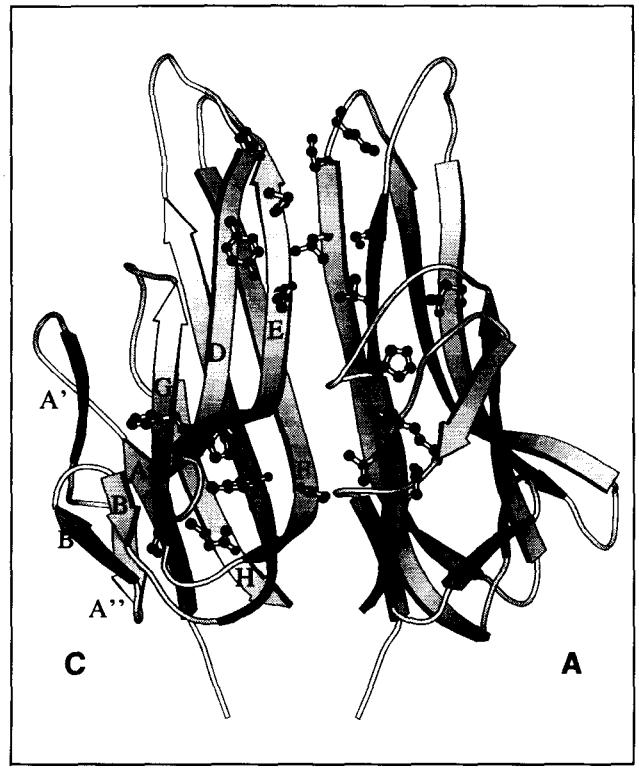

Fig. 5. TNF-β (with the rear subunit removed for clarity) has side chains of the C (left) and A (right) subunits that interact with the TNFr2 receptor.

All members of the TNF family so far described are type II membrane proteins with amino-terminal hydrophobic anchor sequences. TNF-β is an exception, in that it is exported by a classical signal sequence-directed mechanism. Membrane-bound forms of TNF-β are presented on the surface of T lymphocytes, however, in association with a type II membrane protein of the TNF family called lymphotoxin-β [38]. This protein appears to form heterotrimers with TNF-β, predominantly with a 2 : 1 stoichiometry. The function of these heterotrimers in signal transduction or its regulation is unknown. From a structural standpoint, this is a fascinating example of quasi-equivalence in the generation of a symmetric subunit interface.

TNF-β receptor interactions

In the structure of the TNF-β–TNFr1 complex determined by Banner *et al.* [39••], the four domains of the receptor are arranged end to end, forming a rod-like molecule. Although there is little sequence identity between these CRDs, they are quite similar in tertiary structure. Each CRD comprises a pair of antiparallel strand-loop-strand-loop-strand motifs related by a twofold axis (Fig. 6) [31•]. Intron/exon boundaries separating these motifs within gene segments that encode single CRDs, and amino acid sequence homology within repeats, suggest that the domain structure may have arisen through gene duplication (Fig. 6) [31•]. The first three CRDs in the complex are well ordered, and ~50% of the residues in each CRD are structurally conserved. The 10 ordered intradomain disulfides are arrayed like the rungs of a ladder, evenly spaced up and down the long axis of the elongated (~100 Å) monomer. The few residues that are identically conserved among the CRDs have polar or hydrophobic contacts that stabilize the CRD cores (Fig. 6).

The complex consists of three receptors that all interact with a single TNF-β trimer, each receptor molecule being nestled in the crevice formed by adjacent subunits with the long axis of the receptor approximately parallel to the threefold axis of the trimer. The tip of the cone-shaped TNF-β trimer (opposite the membrane anchor) points toward the amino-terminal CRD, nearest the membrane attachment site of the receptor, an orientation optimal for receptor engagement by membrane-anchored forms of TNF [25].

Each receptor makes contact with only two molecules of the TNF-β trimer, forming a 500–600 Å^2 interface with each (Fig. 6). The second CRD of the receptor makes virtually all of the contacts with adjacent TNF-β subunits; the third CRD contributes only a few residues to the interaction. CRD1 and 4 do not interact with the ligand at all in the TNFr1–TNF-β complex. Studies with CRD deletion mutants of TNFr1 [40] gave the seemingly contradictory result that elimination of CRD1 is accompanied by a complete loss of TNF-α binding capability (deletion of CRD4 only weakened binding). In these studies, however, the truncated TNFr segments are joined at their amino termini to immunoglobulin heavy-chain domains to promote dimerization. In this type of construct, the CRD2 is shifted ~30 Å closer to the dimerization domain, and thus cannot be expected to reach its binding site on the TNF trimer.

The twofold topological modularity of the CRD is functionally recapitulated in the TNF-β-receptor interaction; motif 1 of CRD2 forms most of its contacts with one TNF-α subunit, whereas the second motif interacts primarily with the adjacent subunit (Figs 5a, 6). Most of the contacting residues of the ligand lie at the end of β-strands or within loop segments at the subunit interface (Fig. 6). Lymphotoxin undergoes no global conformational changes on binding to the receptors, only local rearrangements, particularly in the DE and A′A′ receptor-binding loops.

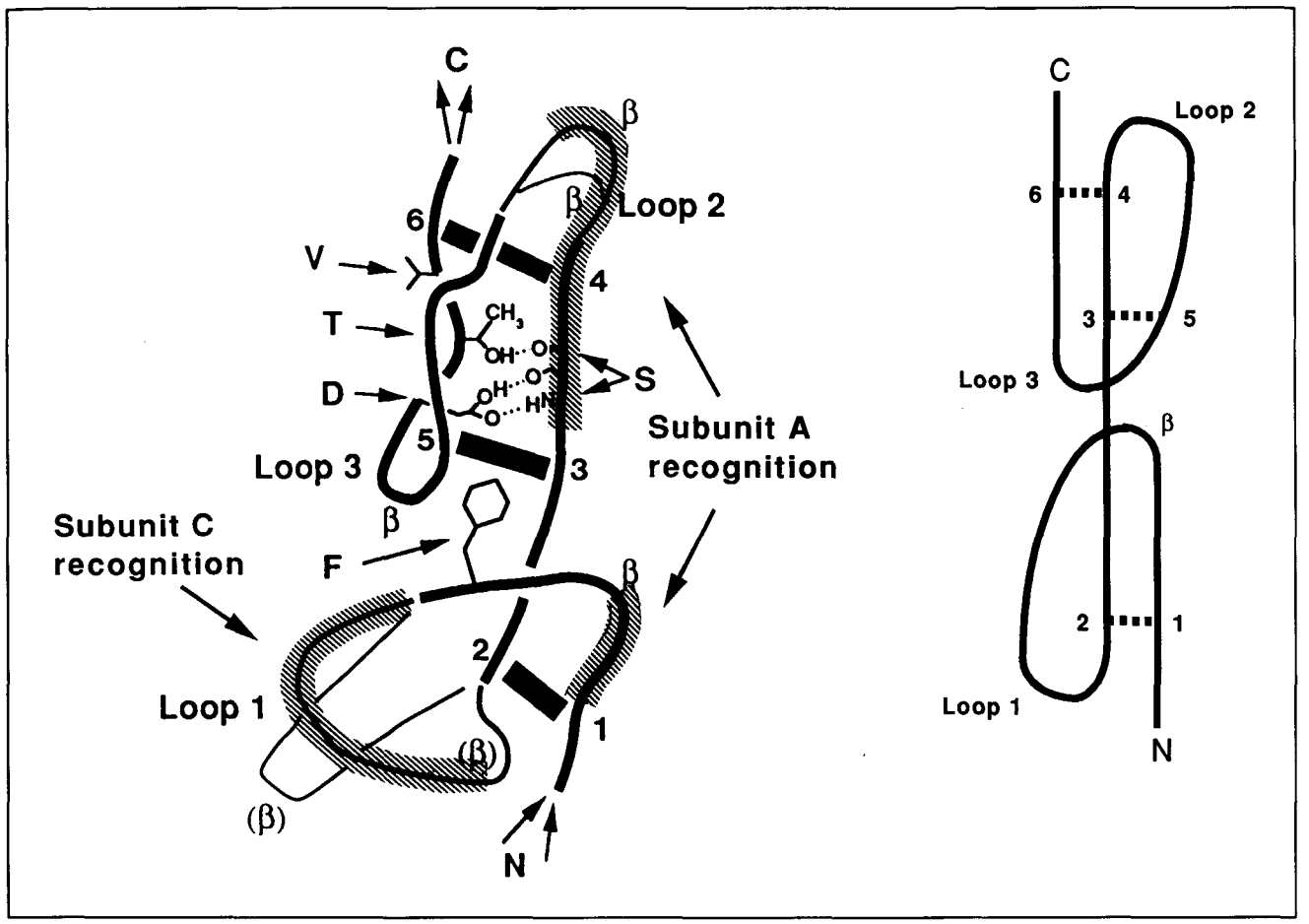

Fig. 6. **(a)** Trace of the CRD2 domain of TNFr2, adapted from Banner *et al.* [39••]. The figure shows three disulfide bridges, represented by very thick lines, and structurally conserved regions, represented by thick lines. Alternative conformations of loop 1 and loop 2 in CDR2 and 3 are shown. Invariant residues that contribute to the stability of the fold are also shown. Matched segments show the parts of CDR2 that interact with subunit C or subunit A of TNF-β (Fig. 5). **(b)** A schematic view of a CRD, shown as a topological repeat dyad symmetry.

It is not at all obvious that the TNF-α–TNFr1 complex is structurally identical to that with TNF-β. Of the 23 residues in TNF-β that bury more than 20 Å^2 of surface area in complex formation, only four are conserved in the primary sequence of human TNF-α. In contrast, 15 of these contact residues are identical among four species variants of TNF-β [41]. Point mutagenesis experiments indicate that residues in the AA′ and DE loops in both TNF-α and TNF-β are involved in receptor contacts [42–44], but it is unlikely that the receptor–ligand contacts are directly comparable. The same receptor might engage different members of the TNF family by using alternative CRD as recognition modules. The primary, almost exclusive, function of CRD2 in the interaction with TNF-β lends some credence to this notion.

The TNF–receptor complex contrasts in interesting ways with that between GH and its receptor. The basic difference is that GH is an asymmetric ligand that binds at the interface between two receptor subunits, whereas the TNF receptor is an asymmetric monomer that binds at the interface between two symmetry-related ligands in the TNF trimer. Whereas GH destroys the dyad symmetry of the receptor, the TNF receptor preserves the trimeric symmetry of the ligand. Solution studies demonstrate that the stoichiometry of the TNF (trimer)–receptor complex is between 1 : 2 and 1 : 3 [45,46], although biological activity appears to require only two receptors bound [47]. In contrast to the GH–receptor interaction, there is no evidence for a sequential mechanism in the binding of receptors to TNF trimers. As a consequence of the threefold symmetry of the TNF trimer, the three intersubunit TNF contact surfaces are identical. The extracellular domains of three receptors within the complex do not contact each other, and trimers do not change conformation with receptor binding. Thus, in accord with the binding data, there is no apparent structural basis for cooperative binding of receptor monomers to TNF trimers. The independence of the receptor-binding sites on the TNF trimer suggests that heterologous receptor–TNF complexes may form *in vivo*. For example, a TNF-α trimer might engage one TNFr1 receptor and two TNFr2 molecules. This type of heterologous interaction may provide a simple mechanism for modulated (depending on the stoichiometry of the species in the complex) cross-talk between receptors.

Species selectivity and receptor specificity

Although native human TNF-α recognizes both the human TNFr1 and TNFr2 receptors, human TNF-α exhibits high affinity only to the mouse TNFr1 receptor. Human TNF-α has recently been exploited as a useful reagent in attempts to define the separate activities of the two receptors in murine cell lines [32,33], although no consensus on the cellular roles of the two receptors has yet emerged. Receptor-selective TNF may prove to be of pharmacological value, as there is some evidence that the larger TNFr2 promotes the systemic cytotoxicity of TNF on non-transformed cells. Indeed, human TNF-α is 50-fold less toxic than murine TNF-α in mice [48•]. Van Ostade *et al.* [48•] have described two human TNF-α mutants that bind the TNFr1 receptor more tightly than TNFr2. The substitutions are both located on the extended A′ segment between the A and A′ strands that form a major point of contact with the receptor CRD2.

Conclusions

In the two examples of receptor–cytokine engagement discussed here, symmetry (or quasisymmetry) is an important element of recognition and binding. Receptors are activated by aggregation, which can be achieved through isologous interactions among symmetry-related ligands, as in TNF, or receptors, as for the hematopoietic growth factors.

The two classes of cytokines that have been discussed have each maintained a distinctive molecular scaffold which supports variable, and broadly divergent, binding surfaces. In contrast to the evolutionally more stolid enzymes, which must maintain unique catalytic stereochemistry while admitting modest changes in substrate specificity or stability, signaling molecules appear to have evolved very rapidly. Relative to metabolic pathways, neuroendocrine and immune regulatory systems diverge rapidly, and continually produce sets of novel signaling molecules with unique specificities. Different strategies have been developed to achieve this. Simple diversity is one, but there are others, for example alternative RNA-splicing mechanisms are exploited to create fetal growth factor receptors with different specificities [49]. The complexity of the cellular immune system appears to require cytokines and receptors with multiple specificities; for example, TNF engages at least two different receptors, each of which in turn can recognize two or more different ligands. Structural studies of anti-idiotypic antibodies demonstrate that a binding surface can recognize common determinants in different and complex epitopes [50]. The cytokines and their receptors also make use of this mechanism.

Acknowledgements

Work at the DNAX Research Institute is supported by Schering Plough. We are grateful to our colleagues who gave us access to their atomic coordinates before public release.

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