ARTICLE

The Galvanization of Biology: A Growing Appreciation for the Roles of Zinc

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Zinc ions are key structural components of a large number of proteins. The binding of zinc stabilizes the folded conformations of domains so that they may facilitate interactions between the proteins and other macromolecules such as DNA. The modular nature of some of these zinc-containing proteins has allowed the rational design of site-specific DNA binding proteins. The ability of zinc to be bound specifically within a range of tetrahedral sites appears to be responsible for the evolution of the wide range of zinc-stabilized structural domains now known to exist. The lack of redox activity for the zinc ion and its binding and exchange kinetics also may be important in the use of zinc for specific functional roles.

Almost all biological processes involve specific interactions between macromolecules. These interactions depend on specific structural domains within the macromolecules. In some cases, the polypeptides that correspond to these domains fold up into the appropriate structures autonomously, but this requires that they have a certain length, typically at least 50 amino acid residues. In recent years, a growing number of domains have been found that are too small to fold by themselves but fold stably when stabilized by bound zinc ions. Because zinc in an appropriate environment can form structure-stabilizing cross-links without introducing undesired chemical reactivity, this ion seems very well suited to this structural role.

Zinc has been known to be an essential trace element in eukaryotes for more than a century (*1*). There are 2 to 3 g of zinc in adult humans, making it one of the most prevalent "trace" elements. However, no specific biological role for zinc was established until 1940, when it was shown to be required for the catalytic activity of carbonic anhydrase (*2*). In the following five decades, the number of known zinc-containing enzymes has mushroomed to more than 300, many of which have been characterized in some detail (*3*). In most of these enzymes, zinc is directly involved in catalysis, interacting with the substrate molecules undergoing transformation. However, in a few enzymes zinc plays a purely structural role. The prototypical example is *Escherichia coli* aspartate transcarbamoylase in which the regulatory subunits contain bound zinc (*4*). Removal of the zinc leads to dissociation of the regulatory subunits from the catalytic subunits with a loss of allosteric regulation but no loss of catalytic activity.

A structural role for zinc in transcription factors was first proposed in 1983 for the protein transcription factor IIIA (TFIIIA) (*5*). Subsequent analysis revealed the presence of small zinc-based domains (termed "zinc fingers") in TFIIIA and in a wide variety of other proteins involved in gene regulation (*6*). Over the past decade, more than 10 classes of such zinc-based domains have been discovered and biochemically characterized (*7*). It has been estimated that proteins containing domains of the type found in TFIIIA may constitute up to 1% of all human gene products (*8*). Included in this group is the protein WT1, mutations in which play a role in the development of Wilms tumor (*9*). The other classes also represent large protein families including the steroid–thyroid hormone receptor superfamily (*10*) and the RING finger protein family (*11*), which includes the breast and ovarian cancer susceptibility gene *BRCA1* (*12*). Furthermore, structural zinc ions have been found in other important proteins and protein complexes including the tumor suppressor p53 (*13*) and the human growth hormone–prolactin receptor complex in which the zinc forms a bridge between the hormone and the receptor (*14*). The protein families in this apparently disparate group have in common tetrahedral zinc-binding sites with four ligands from the side chains of cysteine, histidine, and occasionally aspartate or glutamate. In this article we focus on systems in which zinc plays such structural roles and on the properties of zinc that account for its use in these roles.

Cys_2His_2 Zinc Finger Proteins

Zinc finger domains of the Cys_2His_2 type, initially identified in TFIIIA, appear to represent the most abundant DNA binding motif in eukaryotic transcription factors with more than 1300 sequences reported by October 1991 (*15*) and several thousand known at present. These domains are characterized by tandem arrays of sequences that approximate the form (Tyr,Phe)-X-Cys-X_{2-4}-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-5}-His where X represents more variable amino acids. The structure of each of these zinc finger domains consists of two antiparallel β strands followed by an α helix (*16*) (Fig. 1). This three-dimensional structure nicely accounts for the conserved sequence features: The cysteine and histidine side chains coordinate the zinc, and the three other conserved residues pack to form a hydrophobic core adjacent to the metal coordination unit.

In many cases, proteins containing these domains have been shown to interact with DNA in a sequence-specific fashion. The initial structural information about these interactions came from the crystal structure of the three zinc finger domains from the mouse transcription factor Zif268 bound to a DNA target site (*17*). The zinc fingers in this complex lie in the major groove of the double helix and interact with the DNA bases through amino acid side chains at positions –1, 2, 3, and 6 (hereafter, referred to as the contact residues) with respect to the start of the helical region of each domain. A schematic view of the structure depicting these interactions is shown in Fig. 2. The orientations of the zinc finger domains with respect to the DNA are nearly

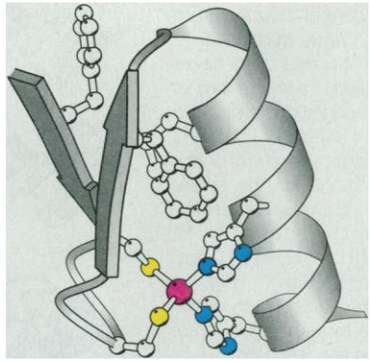

Fig. 1. A schematic view (*56*) of the structure (*17*) of a Cys_2His_2 zinc finger domain. The side chains of the most conserved amino acids (Phe, Phe, and Leu) form a core within the structure that stabilizes the folded conformation in the presence of bound zinc. The remaining amino acids can vary widely to mediate interactions with other macromolecules. Coloring scheme: blue, nitrogen; yellow, sulfur; and pink, zinc.

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identical, and each domain contacts a contiguous 3–base pair subsite, with the majority of the base-specific contacts directed to one DNA strand. There are few interdomain interactions, and the DNA recognition by each zinc finger appears to be largely independent of the other domains.

The interactions observed in the Zif268-DNA complex can be used to rationalize the binding site specificities of several other zinc finger proteins including the human transcription factor SP1 and the yeast transcription factor Adr1p (*18*). These observations have been supported by mutational studies involving the contact residues, some of which resulted in proteins with altered DNA binding specificities (*19*). The binding sites can be successfully rationalized for proteins with guanine-rich binding sites in which the guanine bases are often recognized through their interactions with arginine. However, for a number of other zinc finger proteins, the sequences of the known DNA binding sites cannot be easily reconciled with the available structural information. The elucidation of a more complete set of recognition rules capable of predicting binding sites from amino acid sequences remains a highly desirable goal (*20*). The determination of the structures of complexes involving the DNA binding domains of the human oncogene *GLI* (*21*) and the *Drosophila* regulatory protein tramtrack (*22*) has revealed some additional complexity compared with the Zif268 structure; these structures may be useful in the development of more comprehensive rules.

Considerable work has been directed toward the converse problem, namely that of designing a zinc finger protein that will bind specifically to a desired target DNA sequence. Desjarlais and Berg designed zinc finger proteins by mixing and matching domains for which the apparent three-base subsite specificity had been previously determined (*23*). Furthermore, a consensus sequence that had been shown to form stable zinc finger units (*24*) was used for the framework of each domain with only the contact residues adjusted to dictate the DNA binding specificity. In the first experiment, domains that had been characterized in the context of the middle of the three domains of SP1 were combined to generate a protein that was expected to bind to the sequence 5′-GGG GCG GCT-3′ as illustrated in Fig. 3. Subsequent characterization of the protein revealed that the predicted site is the optimal binding site and the protein-DNA complex has a dissociation constant of approximately 2 nM. This success indicates that it is possible to rationally design zinc finger proteins that recognize a limited subset of potential binding sites.

An alternative approach involves the use of a selection rather than a design strategy. The use of phage display methods for zinc finger proteins has been reported by several different laboratories (*25*, *26*). This scheme uses the expression of zinc finger proteins that include partially randomized recognition helices as part of a phage coat protein for selection for binding to a given DNA sequence. This technique was used to identify zinc fingers for a number of 3–base pair subsites. A disadvantage of this method

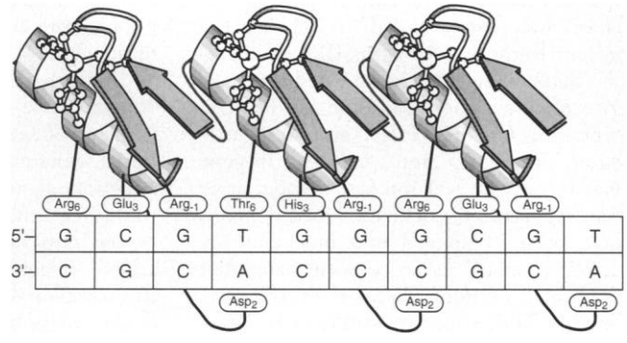

Fig. 2. A schematic view (*56*) of the structure of the Zif268-DNA complex (*17*). The three zinc finger domains are shown, and the juxtaposition of contact residues and DNA bases are indicated. In some cases, these correspond to specific hydrogen-bonding interactions, whereas in others they represent less direct interactions. The subscript numbers indicate the position of the contact residues with respect to the start of the helical region of the respective zinc finger domain.

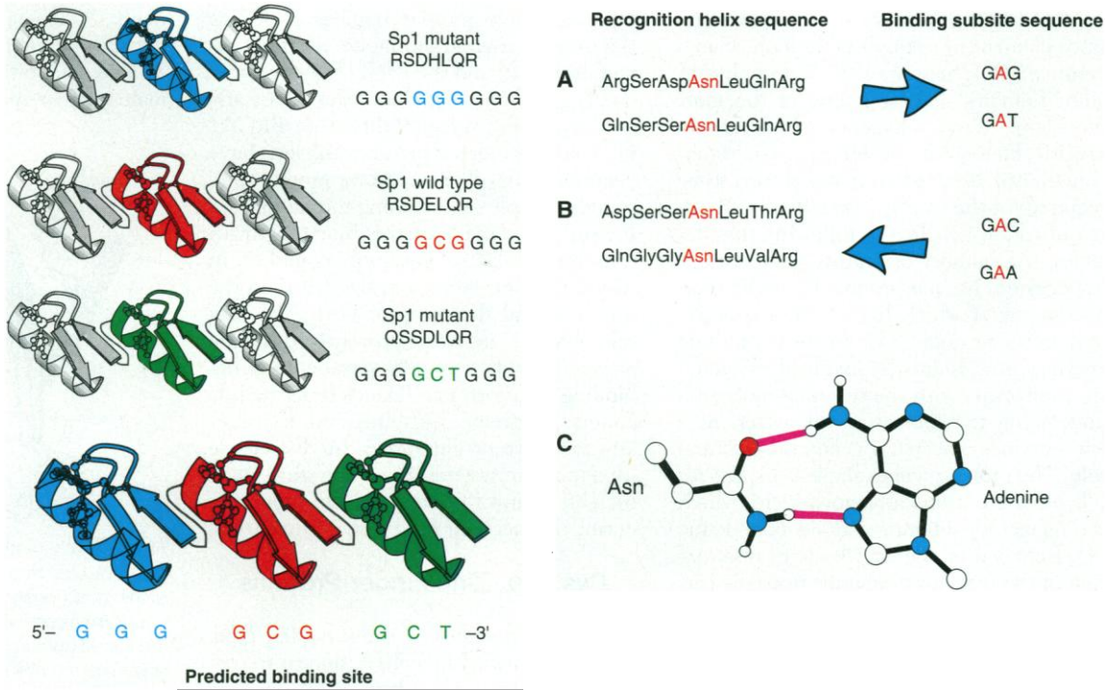

Fig. 3 (left). The design of a sequence-specific DNA binding protein by combination of previously characterized zinc finger modules (*23*). Three domains, studied in the context of the central domain from SP1, were combined to form a novel zinc finger protein. The sequences of the recognition helices and their preferred binding subsites are shown. Abbreviations for the amino acid residues are as follows: D, Asp; E, Glu; H, His; L, Leu; Q, Gln; R, Arg; and S, Ser. **Fig. 4 (right).** Recognition of adenine by asparagine (Asn) in position 3 of a zinc finger domain investigated by different approaches. The convergence of these approaches indicates that these Asn-adenine interactions could be one component of a useful recognition code. (**A**) Determination of binding sites for designed proteins (*19*). (**B**) Selection of binding proteins for given sites with phage display methods (*25*). (**C**) The interaction between Asn and adenine observed crystallographically (*22*) with hydrogen bonds shown in pink.

is that it selects proteins that have the highest overall affinity for DNA rather than those that exhibit highly specific DNA binding. However, complementary methods for rapidly assessing the binding specificity of a given protein have been developed (*27*). Phage display methods have been used to generate a three-domain peptide that recognizes a unique sequence present at a gene translocation (*28*). Proteins containing the selected three-domain unit were found to bind to the desired sequence both in vitro and, remarkably, to repress gene transcription under appropriate conditions in vivo (*28*). In some cases, agreement between the design and selection approaches has been observed. For example, mutants with asparagine at position 3 in the middle domain of SP1 were characterized and found to recognize adenine in the center of the recognition site (*19*). Phage display experiments with mutants of Zif268 on sites containing adenine in this position resulted in recurrent selection of asparagine at position 3 (*25*). The structural basis for this correlation has been revealed in the structure of the two–zinc finger domain fragment from the protein tramtrack bound to DNA (*22*), as summarized in Fig. 4.

The progress toward understanding the rules of how zinc finger proteins interact with duplex DNA masks the fact that the true biochemical role of most zinc finger proteins is not well understood. Some of the proteins do, indeed, act as transcription factors through interactions with double-stranded DNA. However, for other proteins, interactions with other nucleic acids must also be considered. For example, TFIIIA was first identified not as a transcription factor but as a protein that binds 5S RNA in immature *Xenopus* oocytes (*29*). A related protein from the same source, p43, binds 5S RNA, but unlike TFIIIA, it does not appear to bind DNA (*30*). Thus, some zinc finger proteins may function through specific interactions with RNA. More recently it was discovered that certain zinc finger proteins, including SP1 and a designed finger protein, recognize DNA-RNA hybrids in a sequence-dependent manner (*31*). Strikingly, the binding affinities to the hybrids appear to be equal to or even higher than those to the optimal DNA sites for these zinc finger proteins. Because DNA-RNA hybrids are formed during a number of biological processes including gene transcription, DNA replication, and retroviral reverse transcription, this observation suggests that zinc finger proteins could function by binding to DNA-RNA hybrids in vivo. The potential of zinc finger proteins to act by binding to DNA, RNA, heteroduplexes, and even macromolecules other than nucleic acids may allow them to participate in a wide range of biological processes.

Structurally and Functionally Diverse Zinc-Binding Domains

Shortly after the discovery of the Cys_2His_2 zinc-binding domains in TFIIIA and related proteins, other sequences were noted that had the potential to form zinc finger domains (*32*). In some cases, recurring patterns of cysteine and histidine residues had been discovered previously but their potential for forming metal-binding domains had not been noted. The CCHC-box sequences from retroviral nucleocapsid proteins represent one such example. The proteins contain either one or two sequences of the form Cys-X_2-Cys-X_4-His-X_4-Cys. Once the proposal that these sequences might correspond to metal-binding domains was made, metal binding and structural studies were performed first on peptides corresponding to these regions (*33*), then on entire nucleocapsid proteins (*34*), and finally on intact virus particles (*35*). These studies have indicated that retroviral particles contain large amounts of zinc bound primarily to the nucleocapsid protein and have led to new approaches to retroviral therapy (*36*). In other cases, such as members of the steroid–thyroid hormone receptor superfamily, the amino acid sequences were deduced from complementary DNA clones after the zinc-binding domains in TFIIIA and other proteins had been identified (*10*, *37*). The presence of short stretches of amino acid sequence with a high density of cysteine residues was interpreted to be suggestive of metal-binding domains.

More than 10 different classes of zinc-binding domains have now been identified and at least partially characterized (*7*). These classes are structurally and functionally diverse (Table 1). The structural diversity can be illustrated by comparison of three classes of zinc-binding units, each of which bind two zinc ions: the steroid-thyroid receptors (*10*), the RING finger family (*11*), and the GAL4 family (*38*) (Fig. 5). The steroid-thyroid receptor unit (*39*) comprises two separate but interacting zinc finger domains. Each zinc ion is coordinated by four cysteinate residues with the first, second, third, and fourth cysteines coordinating one zinc and the fifth, sixth, seventh, and eighth coordinating the second. In the RING finger domain (*40*), there are also eight metal-binding residues but the sequences that bind the two metal ions overlap. The first, second, fifth, and sixth metal-binding residues coordinate one zinc, and the third, fourth, seventh, and eighth bind the other. In the GAL4 unit (*38*), the metals are bound through six cysteinate residues with two of these residues bridging and interacting with both metal ions. Thus, despite the common use of two zinc ions, these units are quite different from a structural point of view and almost certainly evolved independently.

Table 1. Selected families of zinc-binding domains. C and H donote Cys and His, and X represents other amino acids. NMR, nuclear magnetic resonance.

Zinc domain type (representative or class)	Approximate consensus sequence	Function	Structure	Selected reference
Cys_2His_2 (TFIIIA)	C-X_{2-4}-C-X_{12}-H-X_{3-5}-H	Nucleic acid binding	NMR, x-ray	(*57*)
Cys_8 (steroid-thyroid receptor)	C-X_2-C-X_{13}-C-X_2-C-X_{15}-C-X_5-C-X_{12}-C-X_4-C	DNA binding, oligomerization	NMR, x-ray	(*10*)
Cys_6 (GAL4)	C-X_2-C-X_6C-X_6-C-X_2-C-X_6-C	DNA binding	NMR, x-ray	(*38*)
$Cys_3HisCys_4$ (RING finger)	C-X_2-C-X_{9-27}-C-X_{1-3}-H-X_{2-3}-C-X_2-C-X_{4-48}-C-X_2-C	Protein-protein interaction? Nucleic acid binding?	NMR	(*40*)
$Cys_2HisCys$ (retroviral nucleocapsid)	C-X_2-C-X_4-H-X_4-C	Single-stranded nucleic acid binding	NMR	(*41*)
$Cys_2HisCys_5$ (LIM domain)	C-X_2-C-X_{17-19}-H-X_2-C-X_2-C-X_2-C-X_{16-20}-C-X_{2-3}-C	Protein-protein interaction, DNA binding?	NMR	(*58*)
Cys_4 (GATA-1)	C-X_2-C-X_{17}-C-X_2-C	DNA binding	NMR	(*59*)
Cys_3His (Nup475)	C-X_{6-14}-C-X_{4-5}-C-X_3-H	Unknown	Unknown	(*60*)
$Cys_4HisCys_3$ (requium)	C-X_2-C-X_{11-21}-C-X_2-C-X_4-H-X_2-C-X_{14-17}-C-X_2-C	Unknown	Unknown	(*61*)

The functional diversity of zinc-binding domains is becoming increasingly apparent as more classes of such domains are identified and characterized. The first classes of proteins containing zinc-binding domains to be extensively characterized (the Cys_2His_2 zinc finger proteins, the steroid–thyroid hormone receptor superfamily, and the GAL4 family) all appear to function through interactions with duplex DNA. However, as discussed above, this may not be true for all family members. Other classes appear to function by participating in other sorts of macromolecular interactions. For example, the retroviral nucleocapsid proteins appear to interact with specialized structures in retroviral RNA (*41*). For other domains such as the RING finger motif, the exact function is yet to be determined. Extensive efforts to identify sequence-specific DNA binding activity for such domains have been unsuccessful, and it now appears most likely that RING finger domains participate in protein-protein interactions (*42*). Knowledge of this functional diversity will aid the appropriate interpretation of the discovery of other potential metal-binding domains; the presence of a zinc finger–like sequence motif does not necessarily indicate that the protein interacts with nucleic acids.

This structural and functional diversity belies common principles that unite these metal-binding domains. The binding of zinc allows relatively short stretches of polypeptide chain to fold into well-defined units that are well-suited to participating in macromolecular interactions. The relatively small sizes of these domains make them unlikely to fold in the absence of the additional energetic effects associated with metal ion binding. Direct experimental studies of some metal-binding domains have revealed that they are, indeed, largely unstructured when metal ions are removed (*43*). This coupling of metal binding and protein folding has a number of chemical and biological consequences that may be of importance in fully understanding the functions of proteins containing these domains.

The Special Properties of Zinc

What properties of zinc allow this ion to play structural roles in such a range of important proteins? Zinc exists as a divalent cation, Zn^{2+}, which has a completely filled *d* shell with 10 *d* electrons. This electron configuration has four important consequences. First, because of the filled *d* shell, Zn^{2+} has no ligand field stabilization energy when coordinated by ligands in any geometry (*44*). For ions with partially filled *d* shells, this electronic energy term can favor certain arrangements of ligands over others. Second, in terms of hard-soft acid-base theory, Zn^{2+} is regarded as a borderline acid (*45*). Because of this, zinc can interact strongly with a variety of ligand types including sulfur from cysteine, nitrogen from histidine, and oxygen from glutamate, aspartate, and water. Third, divalent zinc is not redox active; neither the potential oxidized form, Zn^{3+}, nor the potential reduced form, Zn^{+}, is accessible under physiological conditions. Finally, Zn^{2+} is relatively labile in kinetic terms; it undergoes ligand exchange reactions relatively rapidly (*46*).

How do these properties account for the evolution of the wide variety of metal-binding domains, each of which is specific for binding zinc? This question can be approached from either a chemical or a biological perspective. In chemical terms, specific zinc-binding domains appear to evolve readily. The generation of a tetrahedral site from a combination of cysteine and histidine residues will automatically have a preferential affinity for zinc over most other common metal ions (*47*). Most metal ions with partially filled *d* shells lose ligand field stabilization energy upon going from an octahedral site in aqueous solution to a tetrahedral site in a protein. This effect, combined with the borderline acidity of Zn^{2+}, causes tetrahedral sites with a combination of cysteine and histidine ligands to bind zinc more tightly than other metal ions such as Fe^{2+}, Ni^{2+}, and Co^{2+} by factors of two orders of magnitude or more. Thus, the generation of a protein sequence that has potential metal-binding residues with appropriate spacings is likely to bind zinc with at least modest affinity and specificity, and subsequent functional selection can induce tighter binding and other favorable properties. The generation of sites with four coordinating amino acids leads to coordinative saturation, which suppresses the metal-based hydrolytic chemistry observed in zinc enzymes (which generally have only three coordinating amino acids).

In biological terms, zinc has two properties that make it well suited to its role as a structural element in nucleic acid–binding or other gene regulatory proteins. First, its lack of redox activity may be crucial for such a role. Targeting redox-active metal ions such as copper and iron to key DNA and RNA elements could easily lead to the promotion of radical reactions that result in nucleic acid damage. Indeed, molecules such as bleomycin (*48*) and ferrous EDTA (*49*) have been developed as therapeutic or research tools on the basis of this type of reactivity. Thus, it appears that organisms that developed extensive use of copper or iron "finger" proteins would have been lost to evolutionary history. Indeed, in situations where iron-responsive gene regulation has evolved, the interactions with nucleic acids occur with a metal-free protein, and the binding of iron results in the loss of nucleic acid–binding activity (*50*). Second, the relatively facile ligand exchange reactions of zinc appear to be at least partially responsible for the ease of uptake and release of this metal ion. Although little is known about the mechanisms of zinc ho-

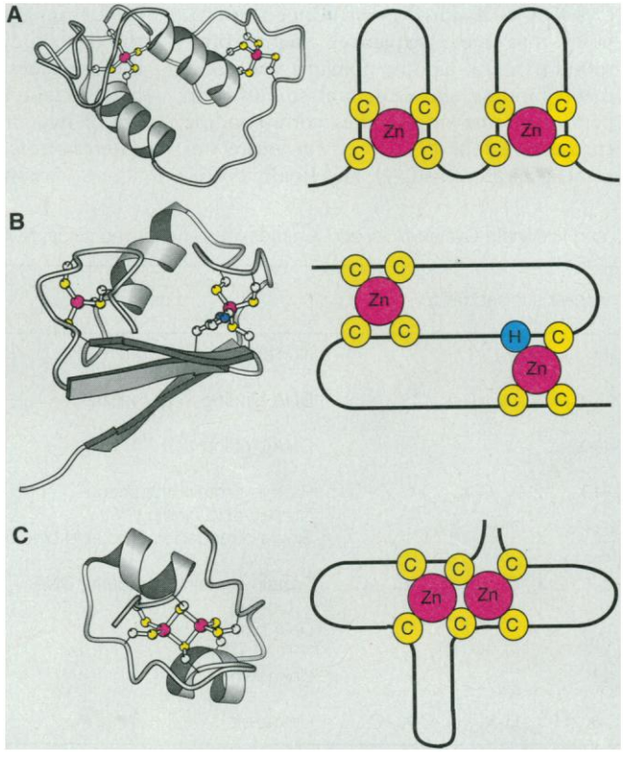

Fig. 5. Structural diversity among zinc-binding domains as illustrated by three classes of domains that each bind two zinc ions. Both schematic three-dimensional structures (*56*) and metal-binding topologies are shown. (**A**) A steroid receptor domain containing two tandem zinc-binding domains (*39*). (**B**) A RING finger domain with overlapping metal-binding sequences (*40*). (**C**) The domain from GAL4 with a binuclear zinc site with bridging cysteine residues (*38*). C, cysteine; H, histidine.

meostasis (as will be discussed below), the maintenance of appropriate levels of zinc under a variety of environmental conditions appears to be achieved, at least for eukaryotes.

Future Prospects

The explosive growth in the number of known zinc-binding domains can be expected to continue as gene and genome sequencing projects advance. It is very likely that many new members of the known classes will be found and new classes of zinc-binding proteins will be discovered. Furthermore, the structural characterization of these domains is also expected to proceed at a rapid pace. The relatively small size of many of these zinc-based domains makes them good candidates for structure determination by nuclear magnetic resonance methods. In parallel with this, x-ray crystallographic methods have proven to be very powerful in elucidating the structures of macromolecular complexes containing these domains.

With the elucidation of the primary and tertiary structures of new domains, additional progress in functional characterization is also to be expected. If domains such as the RING finger domains do, indeed, function in mediating protein-protein interactions, what are their targets? Do these recognize particular families of proteins and peptide targets in a manner that is analogous to the Src homology 2 (SH2) and SH3 domains from signal transduction proteins? With the development of methods such as the yeast two-hybrid system (*51*) and peptide combinatorial library screens (*52*), these questions can now be approached in a direct manner.

The great advancements in our understanding of the occurrence, structures, and functions of these zinc-based domains have served to highlight our ignorance of many other aspects of zinc biochemistry and physiology. Very little is known about how zinc is absorbed, stored, transported, and excreted. It is very likely that zinc concentrations are well controlled both at a cellular and an organismal level, yet the key molecules in such processes are just beginning to be identified and characterized. The characterization of the processes involved in iron homeostasis have revealed a number of fascinating and unexpected mechanisms (*53*). Parallel investigations of zinc homeostatic mechanisms should also be fruitful.

Beyond the questions of zinc homeostasis, the possibility exists that zinc levels are used in vivo in an information-carrying role (*54*). The use of a metal ion in this capacity is well precedented by the role of calcium in a wide variety of signal transduction pathways. Zinc undergoes relatively rapid ligand exchange reactions, exchanging water ligands only one order of magnitude slower than calcium and much faster than many other biologically important ions. Some of the classes of proteins discussed above or other as yet uncharacterized proteins could act to transduce changes in available zinc levels into changes in patterns of gene expression or other forms. The examination of such speculations will depend on the advancement in tools for investigating available zinc concentrations in vivo. Finally, zinc ions may play a different signaling role in the nervous system. Zinc is present in high concentrations in some components of the nervous system, and specialized molecules such as zinc-modulated ion channels and transporters are being discovered and characterized (*55*). It is likely that these processes depend on the interactions of zinc ions with much simpler binding sites, comprised perhaps of properly displayed single cysteine or histidine residues, than the domains that have been the focus here. The elucidation of the interplay between the roles of zinc ions as catalyst components, structural building blocks, and information carriers will provide an exciting story in the field of bioinorganic chemistry.

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