The POZ domain: A conserved protein–protein interaction motif

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We describe a novel zinc finger protein, ZID (zinc finger protein with interaction domain). At its amino terminus ZID contains a 120-amino-acid conserved motif present in a large family of proteins that includes both the otherwise unrelated zinc finger proteins, such as Ttk, GAGA, and ZF5, and a group of poxvirus proteins: We therefore refer to this domain as the POZ (poxvirus and zinc finger) domain. The POZ domains of ZID, Ttk, and GAGA act to inhibit the interaction of their associated finger regions with DNA. This inhibitory effect is not dependent on interactions with other proteins and does not appear dependent on specific interactions between the POZ domain and the finger region. The POZ domain acts as a specific protein–protein interaction domain: The POZ domains of ZID and Ttk can interact with themselves but not with each other, POZ domains from ZF5, or the viral protein SalF17R. However, the POZ domain of GAGA can interact efficiently with the POZ domain of Ttk. In transfection experiments, the ZID POZ domain inhibits DNA binding in NIH-3T3 cells and appears to localize the protein to discrete regions of the nucleus. We discuss the implications of multimerization for the function of POZ domain proteins.

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The large amount of predicted protein sequence data now available has allowed the identification of a number of conserved protein sequence motifs. Because conservation of sequence underlies conservation of structure and function, the presence of a motif of known biochemical function in a predicted protein sequence may provide significant insight into the potential function of that protein. For example, transcription factors contain many different conserved sequence motifs characteristic of different DNA-binding structures, such as the zinc finger, homeo domain, and MCM1, *agamous deficiens*, SRF (MADS) box (for review, see McKnight and Yamamoto 1992); the presence of motifs such as these in the sequences of newly cloned developmentally important genes has enabled their immediate identification as transcription factors in the absence of any direct biochemical data.

Although the majority of the conserved sequence motifs identified in transcription factors is associated with DNA binding, many transcription factors also contain extended motifs known to mediate protein–protein interactions. In transcription factors that bind DNA as dimers, such as those containing leucine zipper or helix–loop–helix motifs, formation of heterodimers allows the generation of a wider spectrum of DNA-binding species than can be achieved by homodimerization, increasing the potential for functional variation (for review, see McKnight and Yamamoto 1992). Perhaps more importantly, regulation of protein–protein interactions may allow regulation of DNA binding, subcellular localization, or transcriptional activation: For example, interaction of the ankyrin repeats of IκB protein with the p65 subunit of NF-κB regulates both subcellular localization and DNA binding and is controlled by phosphorylation and proteolysis (Liou and Baltimore 1993). If a conserved motif can be identified as one mediating protein–protein interactions, it is likely to have important implications for the function of the protein and perhaps its regulation.

Several extended sequence motifs present in transcription factors have no known biochemical function. One such motif is a 120-amino-acid sequence found at the amino termini of many Cys_2–His_2 zinc finger proteins. First identified in the *Drosophila* Tramtrack (Ttk) and Broad Complex (BR-C) zinc finger proteins, this motif is also present in a group of poxvirus proteins and the *kelch* gene product, their putative *Drosophila* cellular homolog, which are not known to bind DNA (DiBello et al. 1991; Koonin et al. 1992; Xue and Cooley 1993). We shall refer to this domain as the POZ (poxvirus and zinc finger) domain. In *Drosophila*, POZ domain proteins have been associated with a variety of processes including nucleosome/chromatin disruption, pattern formation, metamorphosis, oogenesis, and eye and limb development (Harrison and Travers 1990; DiBello et al. 1991; Dorn et al. 1993; Godt et al. 1993; Xiong and Montell 1993; Xue and Cooley 1993; Tsukiyama et al. 1994). In humans, two POZ domain zinc finger genes, PLZF and Bcl6/LAZ3, are associated with chromosomal translocation

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breakpoints in acute promyelocytic leukemia (APL) and non-Hodgkin's lymphoma, respectively (Chen et al. 1993; Kerckaert et al. 1993; Ye et al. 1993; Miki et al. 1994) . In PLZF translocations the PLZF POZ domain and only two of its nine zinc fingers are joined to the retinoic acid receptor (RARα) gene, suggesting that it might be the PLZF POZ domain rather than its zinc finger region, which is instrumental in cellular transformation.

In this paper we report the characterization of a novel POZ domain zinc finger protein, ZID (zinc finger protein with interaction domain). We show that the POZ domain acts to inhibit DNA binding, both in ZID and other POZ domain zinc finger proteins and that it acts as a specific protein–protein interaction domain. These results suggest that protein–protein interactions play an important role in the function of POZ domain proteins.

Results

ZID, a novel zinc finger protein

We set out to isolate cDNAs encoding proteins that interact with the CArG box region of the human skeletal α-actin promoter (Muscat and Kedes 1987). A yeast strain containing a *lacZ* indicator gene controlled by four copies of a CArG box oligonucleotide was transformed with an activator-tagged human cDNA expression library (Dalton and Treisman 1992). A colony color assay for *lacZ* expression identified three colonies expressing CArG box-binding activity, which each contained the same plasmid; conventional techniques were used to isolate additional cDNAs encoding the complete protein. RNase protection analysis indicated that ZID RNA is expressed in various tissues and cell lines. ZID RNA was found at approximately equivalent levels relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA in colon, kidney, lung, skin, spleen, and testes and in the HFF, HeLa, HepG2, and HT1080 cell lines; in brain, ZID RNA was expressed at about a sevenfold higher level (data not shown). The combined cDNA sequence encodes a protein of 424 amino acids that we term ZID (see below; Fig. 1). The sequence includes four C_2–H_2-type zinc finger motifs at its carboxyl terminus. In addition, 120 amino acids at the amino terminus constitute a conserved domain present in a family of proteins that includes many different C_2–H_2 zinc finger proteins and a group of non-zinc finger poxvirus proteins (see Fig. 10, below). The function of this domain, which we term the POZ domain, is the subject of this paper.

Definition of ZID DNA-binding specificity

Preliminary experiments showed that the protein encoded by the original cDNA, Δ139ZID, prepared by cell-

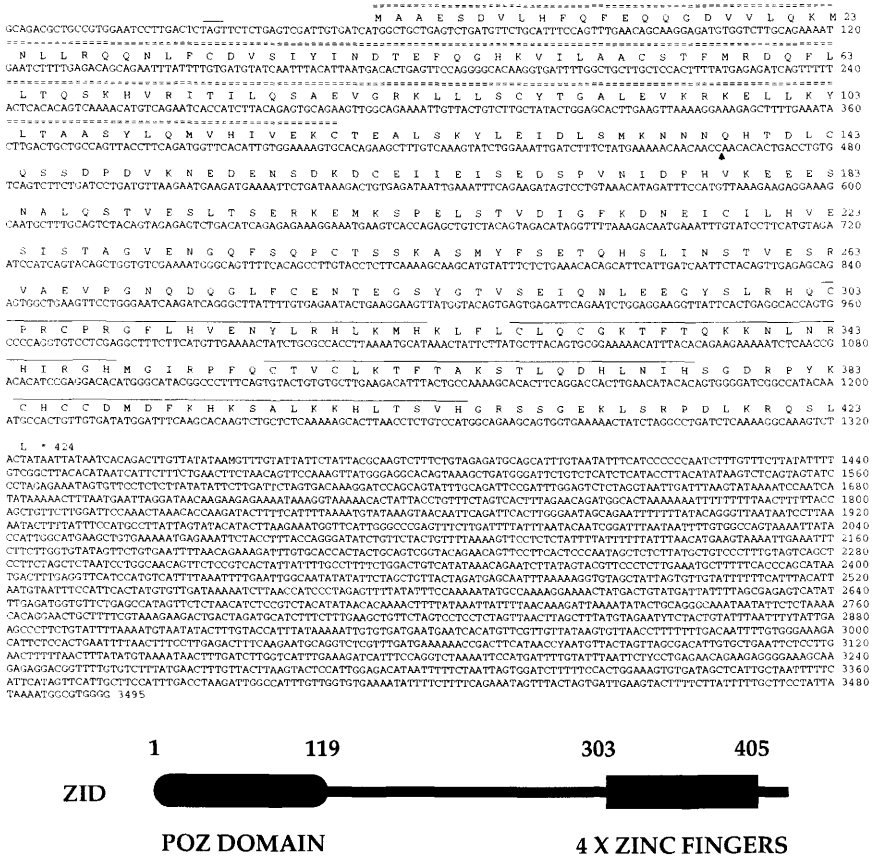

Figure 1. Sequence of the ZID cDNA. The POZ domain is overlined with a double-dashed line; the zinc fingers are overlined with a single solid line. Five sequencing ambiguities are designated with IUPAC code. The in-frame termination codon at the 5′ boundary of the open reading frame is overlined. The POZ domain and the zinc fingers, defined by sequence searches with the tBlastn program (Altschul et al. 1990) are indicated schematically below. Sequences were combined from the following: the original HeLa VP16-tagged cDNA, nucleotides 465 (arrowhead)–1419; two VP16-tagged cDNAs recovered by the PCR, nucleotides 1–464 and 2836–3495; human placental cDNA, nucleotides 523–2835. The sequence is incomplete as it lacks a polyadenylation signal.

free translation, bound only weakly to the CArG box oligonucleotide used for the screen. To define the binding specificity of the ZID zinc fingers in more detail, a binding-site selection experiment was performed. Oligonucleotides selected in this assay contained variants of the consensus sequence (C/T/G)GGCTCYA(T/G)(C/G)(A/G)YC, which mismatches the CArG oliognucleotide at two conserved positions (Fig. 2A). We used various selected oligonucleotide probes in the gel mobility retardation assay to test whether this represents the bona fide ZID DNA-binding consensus. The protein efficiently and specifically bound a probe containing the consensus sequence; binding was insensitive to addition of excess unlabeled mutant consensus oligonucleotide carrying C → G transversions at the two most conserved positions (Fig. 2B, lanes 1–3). DNA-binding analysis with other selected sequences showed that in general the more closely a probe matches the consensus, the more strongly it binds the protein (Fig. 2B, lanes 4–24). The derived consensus therefore accurately reflects ZID zinc finger DNA-binding specificity in vitro. The binding consensus oligonucleotide probe was used to examine HeLa cell extracts for DNA-binding activity; no specific complexes were detectable (data not shown).

The POZ domain inhibits DNA binding

We tested the DNA-binding activity of intact ZID using the gel mobility retardation assay. Surprisingly, intact ZID protein bound the consensus binding site weakly, generating complexes that appear heterogeneous (Fig. 3A, lane 2; see also Fig. 8A, lane 2, below). To map the ZID sequences responsible for this effect, we analyzed a set of ZID deletion derivatives. Precise removal of the entire POZ domain generated proteins that formed discrete high affinity complexes; more extensive amino-terminal deletions did not increase binding further (Fig. 3A, lanes 4–6). Quantitation of the data by PhosphorImager indicated that these deletions increased binding affinity approximately eightfold. In contrast, deletion of either half of the POZ domain or sequences between the POZ domain and the zinc fingers had no effect (Fig. 3A, lanes 3,7,8).

We next examined DNA binding by two other POZ zinc finger proteins, GAGA and Ttk (Harrison and Travers 1990; Soeller et al. 1993). These proteins and truncated derivatives lacking their POZ domains were prepared by cell-free translation, and appropriate DNA probes used to assess DNA binding by the gel mobility retardation assay. Neither protein bound DNA efficiently unless sequences including the POZ domain were removed (Fig. 3A, lanes 9–14). Taken together, these data suggest that inhibition of DNA binding in vitro is a general property of POZ domains and is not specific to ZID.

To exclude the possiblity that these results reflect the inability of complexes formed by the intact proteins to enter the gel, we also analyzed DNA binding using an indirect DNA immunoprecipitation assay. For this experiment, derivatives of the proteins including the

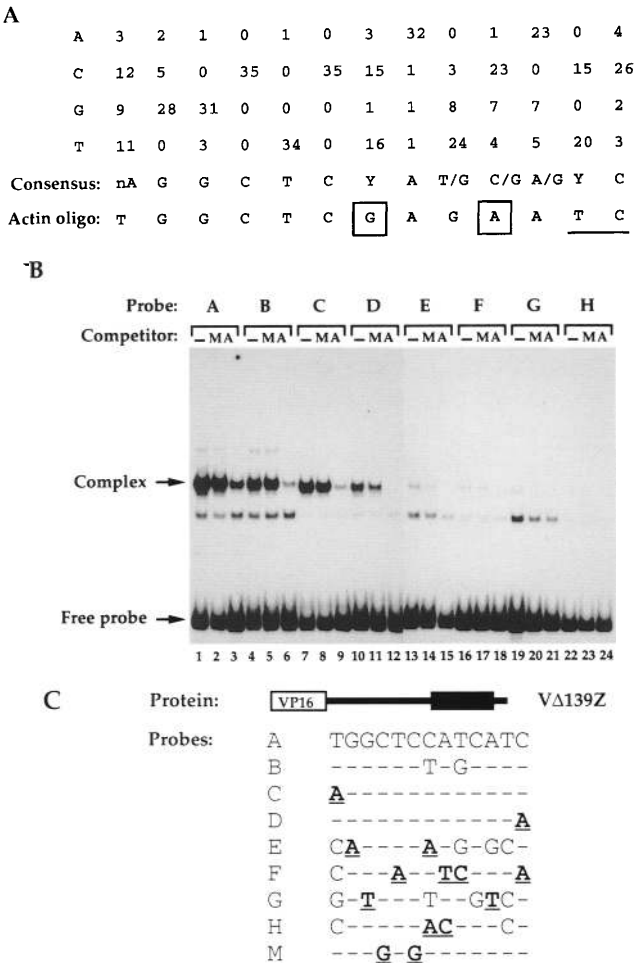

Figure 2. ZID DNA-binding specificity. (*A*) Sequences of 35 oligonucleotides recovered by binding site selection were aligned with respect to the ZID DNA-binding site, and nucleotide frequency at each position is indicated. Of the 46 oligonucleotides sequenced, 40 contained consensus matches (5 overlapping the primer sequence) and 6 contained no match. Below the derived consensus is part of the sequence of the skeletal α-actin oligonucleotide used to select the ZID cDNA: (nA) C, G, or T; the boxes indicate mismatches to the consensus, and the underlined base pairs are from linker sequence. (*B*) Binding of the ZID derivative VΔ139Z to various selected oligonucleotide probes [A–H; see (*C*)] was tested by gel mobility retardation assay. (Lanes *1,4,7,10,13,16,19,22*) No competitor oligonucleotide; (lanes *2,5,8,11,14,17,20,23*) 300-fold excess unlabeled mutant oligonucleotide M; (lanes *3,6,9,12,15,18,21,24*) 300-fold excess unlabeled consensus oligonucleotide A. Positions of the free probe and the specific VΔ139Z–oligonucleotide complex are indicated; the upper complex in lanes *1*, *2*, *4*, and *5* probably arises from a second ZID weak site in these probes. VΔ139Z was used to distinguish the specific complex from the faster migrating complex, which is not specific to the ZID site. (*C*) Structure of the VP16–ZID fusion protein, VΔ139Z. Sequence of the consensus oligonucleotide ZID probe A is shown; other probe sequences show only mismatches to the consensus probe (bold type, underlined) and differences from probe A that still match the consensus (normal type). Probes are from the binding-site selection, and the context of the binding sites therefore differs in each. Competing synthetic oligonucleotides A and M are 21 bp.

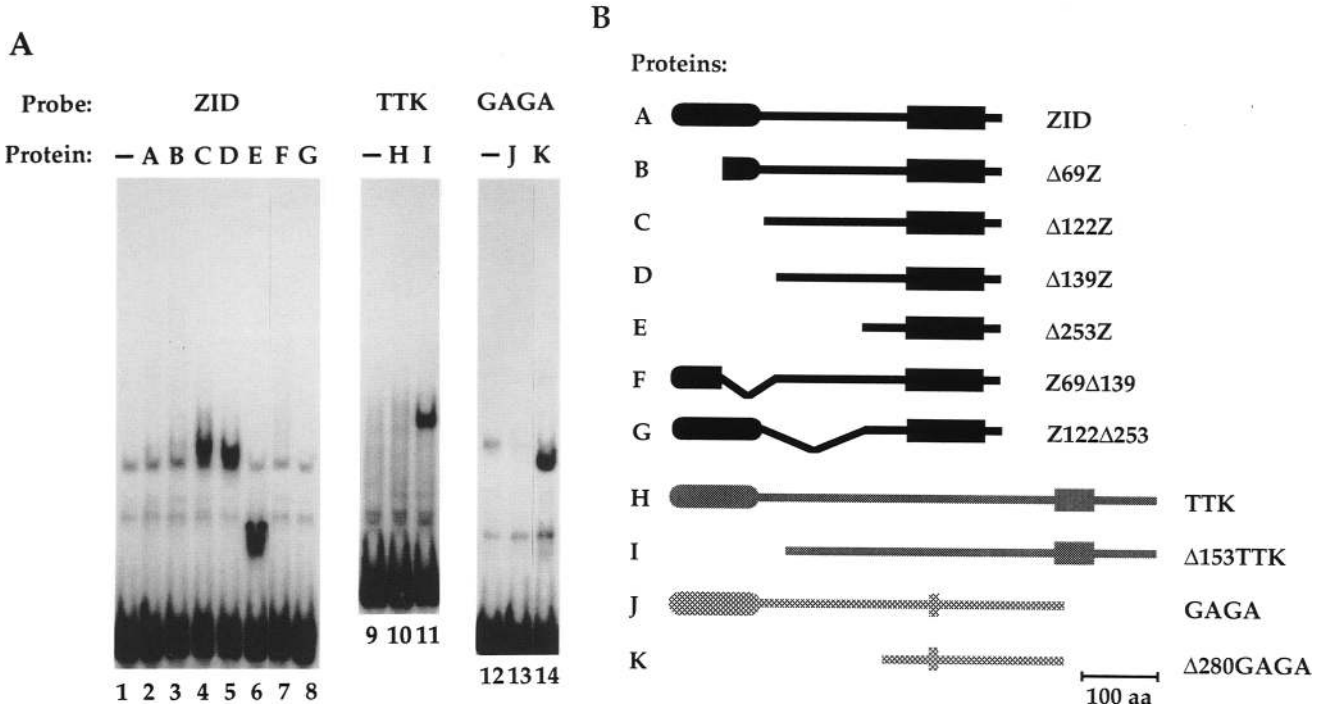

Figure 3. The POZ domain inhibits DNA binding. (*A*) DNA binding by ZID, Ttk, and GAGA proteins and deletion derivatives (diagramed in *B*) was tested using appropriate probes. (Lanes *1,9,12*) Unprogrammed lysate. (*B*) Structures of the proteins tested in *A*. In this and subsequent figures all proteins are drawn to scale, with each parent protein and its derivatives shaded differently, and reference letter code for *A* on the left. POZ domains are shown as extended ovals and DNA-binding domains as rectangles. Protein names are at the *right*: Deletions are denoted by Δ, and numbers indicate the first amino acid that is present in the protein.

epitope tag 9E10 (Evan et al. 1985; Munro and Pelham 1987) at the carboxyl terminus were constructed. Radiolabeled proteins either containing or lacking the amino-terminal POZ domain were incubated with appropriate radiolabeled DNA probes; protein–DNA complexes were recovered by immunoprecipitation and analyzed by SDS-PAGE. Quantitation of these data using the PhosphorImager showed that removal of their POZ domains increases the DNA-binding affinity of ZID and Ttk 5- and 10-fold, respectively (Fig. 4A, lanes 6,7 and 9,10).

The POZ domain inhibits DNA binding by purified ZID

To test whether the inhibitory effect of the POZ domain requires its interaction with other proteins in the reticulocyte lysate, we analyzed DNA binding by purified ZID derivatives. ZID and a derivative lacking the POZ domain, Δ122Z, were expressed in *Escherichia coli* as carboxyl-terminal histidine-tagged fusion proteins, purified from inclusion bodies under denaturing conditions,

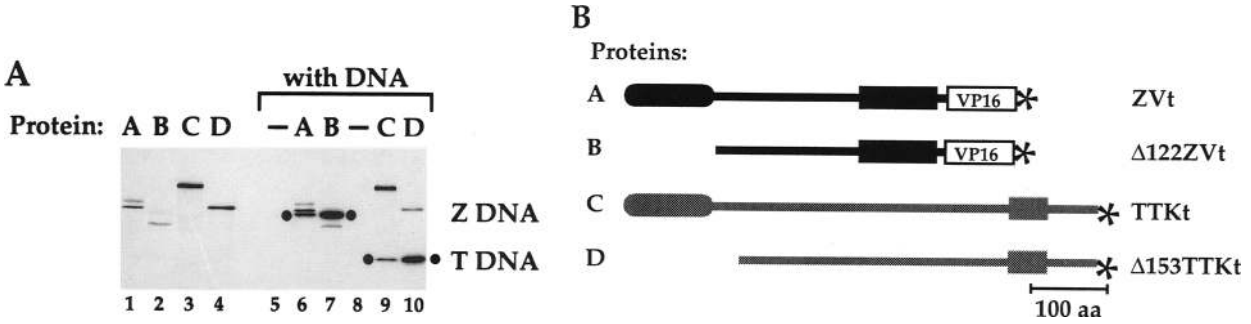

Figure 4. Indirect DNA immunomprecipitation assay of ZID and Ttk DNA binding. (*A*) (Lanes *1–4*) One microliter of 9E10 epitope-tagged VP16–ZID and Ttk derivatives with (lanes *1,3*) and without (lanes *2,4*) the POZ domain fractionated by SDS-PAGE (10% gel): note that ZID proteins migrate as a doublet (lanes *1,2*). (Lanes *5–10*) Five microliter programmed (lanes *6,7,9,10*) or unprogrammed (lanes *5,8*) lysates were incubated with ^{32}P-labeled ZID or Ttk probes and immunoprecipitated with 9E10 antibody, and recovered protein and DNA were fractionated by SDS-PAGE (lanes *5–7*, ZID; lanes *8–10*, Ttk). Position of the recovered DNA is indicated by (●). (*B*) Structures of the proteins used in *A*; the 9E10 epitope is indicated (* and t).

and renatured. Protein Δ122ZH, a polypeptide of 46 kD (Fig. 5A, lane 2), bound DNA efficiently in the gel mobility retardation assay (Fig. 5A, lane 5). Purified full-length ZH protein preparations contained the expected polypeptide of 58 kD, but also always included a species of 44 kD (Fig. 5A, lane 1). Because these proteins were purified under denaturing conditions via a carboxy-terminal histidine tag, the 44-kD protein must represent an amino-terminal truncation lacking the POZ domain. Complexes formed by intact ZH protein would be expected to migrate more slowly than Δ122ZH; however, with full-length ZH preparations only a complex of somewhat greater mobility than Δ122ZH was detected, which presumably represents DNA binding by the proteolytic cleavage product (Fig. 5A, cf. lanes 4 and 5). We conclude that interaction with additional proteins is not required for the inhibition of DNA binding by the POZ domain.

The POZ domain inhibits activity of linked DNA-binding domains

One way in which the ZID POZ domain might inhibit DNA binding is by a specific interaction with the ZID zinc finger region. To test this possibility we examined the DNA-binding properties of chimeric proteins, in which either the ZID POZ domain was replaced by heterologous POZ domains, or its zinc fingers were replaced by those from a non-POZ zinc finger protein (Fig. 6B). POZ domains from the mouse zinc finger protein ZF5 (Numoto et al. 1993) and the vaccinia virus protein SALF17R, which is not known to bind DNA (Howard et al. 1991), both inhibited DNA binding by the ZID zinc fingers; as with ZID, heterogeneous slowly migrating complexes were detectable in both cases (Fig. 6A, lanes 1–6). Moreover, the ZID POZ domain inhibited DNA binding when linked to the zinc finger region of SP1 (Kadonaga et al. 1987), a non-POZ zinc finger protein (Fig. 6A, lanes 7–9).

These experiments suggest that inhibition of DNA binding does not require a specific interaction between the POZ domain and the zinc finger region. We therefore tested whether the ZID POZ domain could interfere with DNA binding when linked to DNA-binding structures other than C_2–H_2 zinc fingers. Three different types of DNA-binding domains were linked to the ZID POZ domain: The Oct-1 POU/homeo domain (Sturm et al. 1988), the serum response factor (SRF) DNA-binding domain (Norman et al. 1988), and the Cys_2–Cys_2 zinc finger domain of RARα (Giguere et al. 1987). These proteins bind DNA as monomers, homodimers, and heterodimers [with retinoid X receptor (RXR)], respectively; the ZID/RARα chimera was examined because it resembles the PLZF–RARα chromosomal translocation (see Discussion). The DNA-binding activity of each chimera was compared with that of an appropriate derivative lacking the POZ domain. In each case, the ZID POZ domain greatly inhibited DNA binding by the chimeric protein (Fig. 6A, lanes 10–24). Taken together, these results imply that the inhibitory effect of the POZ domain on DNA binding does not result from a specific interaction between it and the zinc finger region.

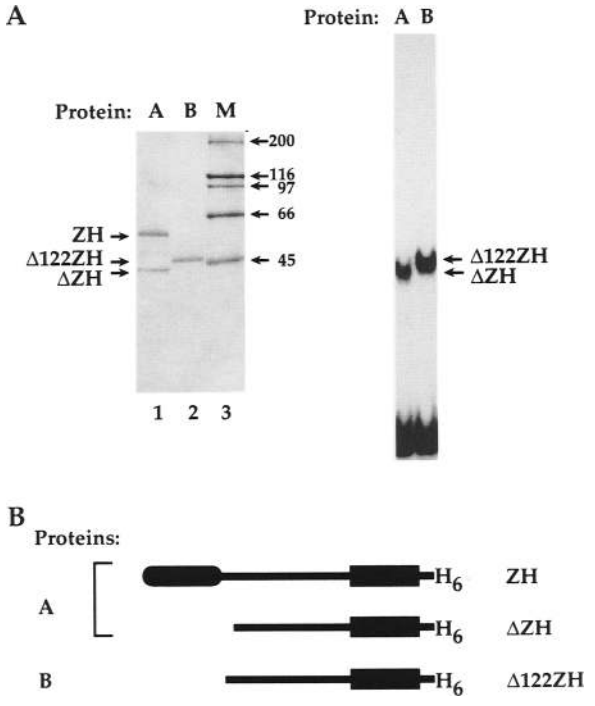

Figure 5. Inhibition of DNA binding by the POZ domain does not require other proteins. (*A*) (Lanes *1–3*) SDS-PAGE analysis of recombinant histidine-tagged ZID derivatives expressed in *E. coli*. (Lane *1*) Preparations of full-length ZID contain a presumed amino-terminal cleavage product (ΔZH) in addition to the intact protein (ZH). (Lane *2*) Δ122ZH; (lane *3*) markers; (lanes *4,5*) gel mobility retardation analysis of full-length ZID (lane *4*) and Δ122ZH (lane *5*). (*B*) Structures of the proteins used in *A*.

The POZ domain is a protein–protein interaction motif

We next considered the possibility that the POZ domain mediates oligomerization, and this somehow prevents access of the zinc fingers to DNA. To test this idea we used an immunoprecipitation assay, in which epitope-tagged and nontagged proteins were produced by cotranslation and immunoprecipitated using the tag antibody. In this assay, efficient coprecipitation of the nontagged protein is indicative of a stable interaction between the proteins. Two carboxy-terminally 9E10 epitope-tagged ZID derivatives were constructed: One comprised the amino-terminal half of ZID, whereas the second was otherwise identical but lacked the POZ domain (Fig. 7B, proteins A and B). Interaction of these proteins with a variety of nontagged ZID derivatives was tested (Fig. 7B, proteins D–I). Coprecipitation of the nontagged protein was only observed in cases where both proteins contain the intact ZID POZ domain (Fig. 7A, cf. lanes 1, 3, and 7); similar results were seen when full-length tagged ZID was used (data not shown). When nontagged and tagged ZID derivatives of differing sizes were mixed together

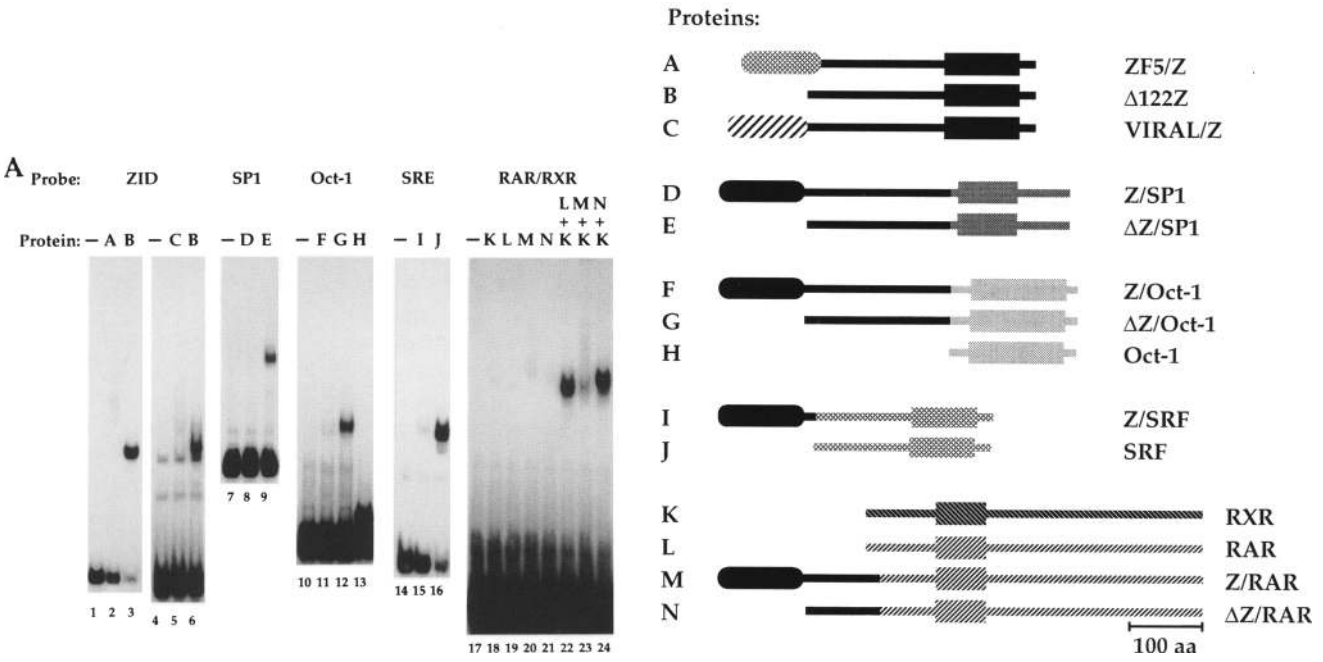

Figure 6. Specificity of POZ domain action. (*A*) DNA binding by the various chimeric proteins shown schematically in *B* was tested using appropriate probes. (Lanes *1–6*) ZID; (lanes *7–9*) SP1; (lanes *10–13*) Oct-1; (lanes *14–16*) SRF; (lanes *17–24*) DR + 5). (lanes *1,4,7,10,14,17*) Unprogrammed lysate (nonspecific complexes vary between different batches). RAR and RXR proteins were mixed after translation; the same result was seen if the proteins were cotranslated (not shown). (*B*) Structures of the proteins assayed in *A*.

rather than cotranslated, coprecipitation was inefficient, indicating that limited exchange took place and the interaction is kinetically stable (data not shown). The ZID POZ domain did not interact with ZID derivatives containing either the amino- or carboxy-terminal portions of the ZID POZ domain or the intact POZ domains from the ZF5, GAGA, or viral SalF17R proteins, showing that the interaction is specific (Fig. 7A, lanes 5,6,8; data not shown).

We next tested whether the Ttk POZ domain behaves similarly to that of ZID. For these experiments we used a Ttk derivative in which its amino-terminal sequences including the POZ domain are linked to a carboxy-terminal 9E10 epitope tag (Fig. 7B, protein C). In the immunoprecipitation assay this protein interacts efficiently with a nontagged Ttk derivative that contains its POZ domain but does not interact with the POZ domains of ZID and ZF5 (Fig. 7A, lanes 9–11; data not shown). In contrast, the Ttk POZ domain interacts with the GAGA POZ domain as efficiently as it interacts with itself (Fig. 7A, lanes 12,13). The strict sequence specificity of these interactions strongly suggests that the proteins produced in vitro are correctly folded. These experiments demonstrate that the POZ domain is a protein–protein interaction domain and imply that it is protein–protein interactions mediated by the POZ domain that result in inhibition of DNA binding.

These observations raise the possibility that the multimeric forms of POZ zinc finger proteins bind specific DNA sequences distinct from those recognized by the finger region alone. However in site selection experiments, the intact proteins select, albeit more slowly, oligonucleotides containing the same consensus sequence selected by the fingers alone (data not shown; see Discussion).

DNA binding by ZID–POZ heterodimers

The experiments described above suggest a model in which efficient access of DNA to the zinc finger region of the protein is sterically hindered in the complex that results from POZ domain interactions. In this case, formation of a mixed complex between ZID and another POZ protein might result in increased accessibility of the ZID zinc fingers to DNA. To test the feasibility of this, we asked whether cotranslation of full-length ZID with partial ZID fragments containing the POZ domain resulted in the activation of ZID DNA-binding activity. Cotranslation of the central non-POZ, non-zinc finger region of ZID with intact ZID has no effect on DNA binding (Fig. 8A, lanes 2,3). In contrast, cotranslation of intact ZID with any one of three proteins containing an intact ZID POZ domain results in generation of discrete complexes, the mobility of which is proportional to the size of the ZID fragment (Fig. 8A, lanes 4,5,7). The DNA-binding activity generated in this fashion is weak compared with that of ZID derivatives lacking the POZ domain and requires the presence of an intact ZID POZ domain in the cotranslated protein (Fig. 8A, lane 8; data not shown). Because the cotranslated ZID derivatives

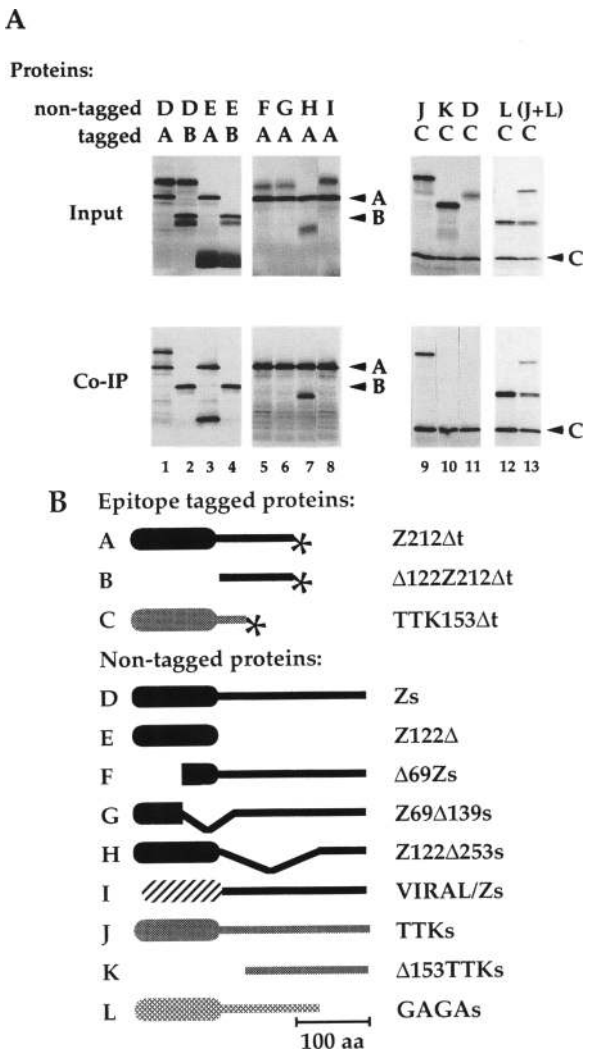

Figure 7. The POZ domain is a specific protein–protein interaction domain. (*A*) (*Top*) 15% SDS-PAGE analysis of 1 μl of [^{35}S] methionine-labeled nontagged and 9E10 epitope-tagged proteins (shown schematically in *B*), produced by cotranslation in the combinations indicated above each lane. Some proteins migrate diffusely due to comigration of lysate proteins. (*Bottom*) SDS-PAGE analysis of proteins recovered from 5 μl of lysate by immunoprecipitation using 9E10 antibody. (*B*) Structures of the proteins tested in *A*. (s) The proteins terminate at amino acid position 308, ZID; 321, Ttk; 245, GAGA.

lack the zinc fingers, it is likely that these complexes represent DNA binding by ZID hetero-oligomers. To examine the stoichiometry of these complexes, we cotranslated intact ZID with two different ZID derivatives containing the POZ domain. Two discrete protein–DNA complexes were generated, corresponding to those seen when the ZID derivatives were cotranslated individually with intact ZID. This suggests that the discrete DNA–binding species is a heterodimer composed of the intact ZID protein and the cotranslated derivative (Fig. 8A, cf. lanes 4–6).

The POZ domain inhibits ZID DNA binding in vivo

Finally, we used transfection experiments to examine the activity of ZID in vivo. Reporter plasmids were constructed in which transcription of the chloramphenicol acetyltransferase (CAT) gene is controlled by one or three copies of the consensus ZID DNA-binding site or a mutated derivative. These reporter plasmids show similar low levels of transcriptional activation upon transfection into NIH-3T3 and HeLa cells, indicating that the ZID binding site does not act as a positive or negative promoter element in these cells (Fig. 9A, lanes 1,3,5; data not shown). Cotransfection with expression plasmids expressing either intact ZID or a derivative lacking the POZ domain had no effect on reporter gene activity (data not shown). In contrast, cotransfection with an expression plasmid encoding VΔ139Z, in which the POZ domain is replaced by the VP16 transcriptional activation domain, efficiently and selectively activated the reporters containing the intact ZID DNA-binding sites (Fig. 9A, lanes 2–6).

Because ZID alone did not appear to activate transcription, we used ZID–VP16 fusion proteins to assay ZID DNA binding in vivo: The VP16 activation domain is

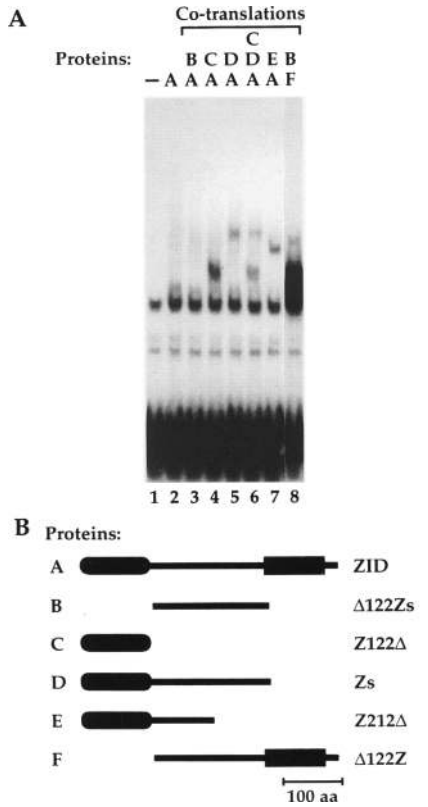

Figure 8. ZID DNA binding can be rescued via dimerization with an isolated POZ domain. (*A*) ZID and its derivatives were produced either alone or by cotranslation in the combinations indicated above each lane. DNA-binding activity was tested by gel mobility retardation assay with a ZID oligonucleotide probe. (Lane *1*) Unprogrammed lysate. (*B*) Structures of the proteins.

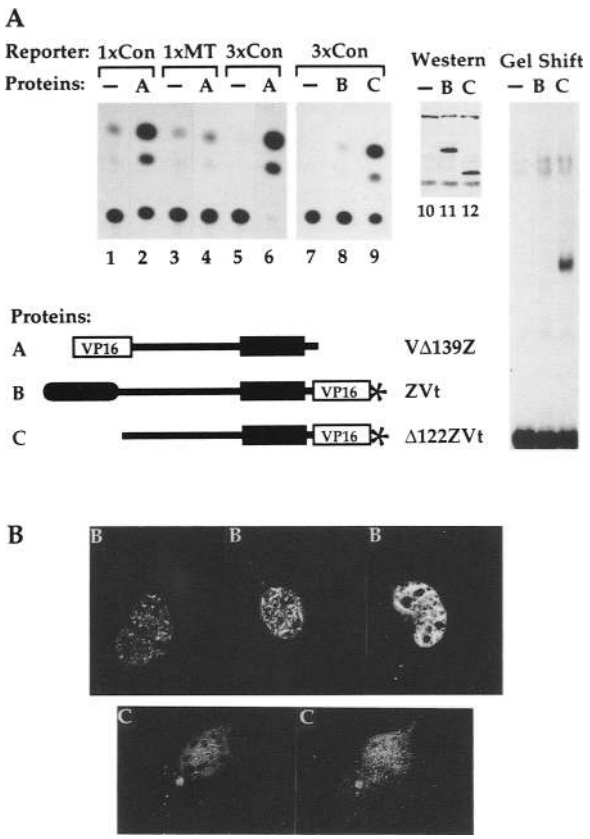

Figure 9. ZID activity and localization in vivo. (*A*) (Lanes *1–9*) Transcriptional activation by ZID–VP16 derivatives. NIH-3T3 cells were transfected with plasmids encoding various derivatives (lanes *1–6*, 1 μg; lanes *7–9*, 0.1 μg) together with CAT reporter plasmids (4 μg) controlled by one or three consensus ZID DNA-binding sites (1XCon and 3XCon) or one mutant ZID DNA-binding site (1XMT). (Lanes *10–15*) Immunoblot (9E10 antibody, lanes *10–12*) and gel mobility retardation analysis (lanes *13–15*) of extracts prepared from cells transfected with ZID–VP16 expression plasmids (5 μg) with probe B (see Fig. 1). Structures of the ZID–VP16 derivatives are shown schematically below; expression vectors were MLVplink (lanes *1–6*) or EF–BOS (lanes *7–15*, and in *B*). (*B*) Immunofluorescence analysis of NIH-3T3 cells transfected with EF–BOS ZID–VP16 expression plasmids, using 9E10 epitope tag antibody. Confocal images: ZVt three different cells, Δ122ZVt same cell upper and lower plane. Protein structures same as above.

constitutively active, and reporter gene activity should therefore reflect the efficiency of DNA binding by different ZID derivatives. The VP16 sequences, together with the 9E10 epitope, were inserted carboxy-terminal to the ZID zinc fingers. Significant reporter gene activation was observed only with the ZID derivative lacking the POZ domain, strongly suggesting that the POZ domain inhibits its DNA binding by ZID in NIH-3T3 cells in vivo (Fig. 9A, lanes 7–9). Control experiments confirmed that both the ZID–VP16 fusion proteins accumulated to similar levels, as assessed by immunoblotting experiments (Fig. 9A, lanes 10–12); in addition, when extracts of transfected cells were analyzed by the gel mobility retardation assay, significant ZID-specific DNA-binding activity was only detectable in extracts from cells expressing the ZID derivative that lacks the POZ domain (Fig. 9A, lanes 13–15). Similar results were obtained in the transcriptional activation assay even when the two fusion proteins were expressed at levels below the detection limits of the immunoblot and DNA-binding assays (data not shown).

We examined the subcellular location of the two ZID derivatives by immunofluorescence. The ZID–VP16 derivative containing the POZ domain is found predominantly in the nucleus, in structures of varying size, which in some cases are visible under phase contrast (Fig. 9B, protein B; data not shown). Deletion of the POZ domain resulted in more diffuse staining in the nucleus and staining to a lesser extent in the cytoplasm (Fig. 9B, protein C). In contrast, cells expressing 9E10-tagged SRF at five times the level of the ZID derivatives show strong but diffuse nuclear staining (data not shown). Thus, the POZ domain is required for efficient nuclear localization and appears to direct ZID to discrete regions of the nucleus.

Discussion

We have characterized a novel zinc finger protein termed ZID. ZID is a member of a large family of proteins that contain a 120-amino-acid sequence motif, which we term the POZ domain. The POZ domain mediates protein oligomerization, and in all POZ proteins examined this interaction prevents high affinity DNA binding. It will be interesting to see whether other conserved motifs found in zinc finger proteins, such as the KRAB and FAX domains, are also involved in protein interactions (Knochel et al. 1989; Bellefroid et al. 1991; Rosati et al. 1991; Pengui et al. 1993).

We know little about the function of the ZID protein itself. Although we can detect ZID-specific RNA in many cell lines and tissues, we are unable to detect ZID-specific DNA-binding activity in cell extracts: This may reflect either failure to translate ZID RNA or, perhaps, POZ-dependent inhibition of ZID DNA-binding activity. It is unlikely that ZID plays a role in skeletal actin gene regulation, as the sequence used to identify ZID cDNAs represents a low affinity and evolutionarily nonconserved ZID binding site; moreover, a search of the sequence data base for conserved matches to the ZID consensus revealed no potential ZID DNA-binding sites of clear biological relevance. Several POZ zinc finger proteins have been proposed to be transcriptional repressors (Harrison and Travers 1990; Read et al. 1992; Brown and Wu 1993; Numoto et al. 1993; Xiong and Montell 1993): However, although an attractive view is that POZ domains act to inhibit transcriptional activation in vivo, we observed neither activation nor repression of transcription by ZID. Further study of the ZID protein in vivo will be required to provide insight into its biological role.

The POZ domain

The sequences of 28 POZ domains present in the data base at the time of submission are shown in Fig. 10. The first POZ domain proteins identified were encoded by poxviruses (Upton and McFadden 1986); however, the POZ domain was only recognized as a discrete protein sequence motif when sequences of the *Drosophila* Ttk and BR-C proteins were compared with those of the viral proteins (DiBello et al. 1991; Koonin et al. 1992). We have used the term POZ in recognition of these prototypical proteins; however, the term BTB (Broad Complex, Tramtrack, and Bric-a-brac) has been used for *Drosophila* proteins (Godt et al. 1993), and the amino-terminal 41 amino acids have been referred to as the ZiN [zinc finger N (amino)-terminal] domain (Numoto et al. 1993). The majority of POZ domain proteins, which appear restricted to multicellular eukaryotes, fall into two classes. The first class comprises C_2–H_2 zinc finger proteins with amino-terminal POZ domains. The second class comprises the poxvirus proteins, the *Drosophila kelch* protein, and the *Caenorhabditis elegans* T16H12.6 predicted protein: These proteins show sequence similarity thoughout their length and include a repeated carboxy-terminal motif of unknown function (Koonin et al. 1992; Senkevich et al. 1993; Xue and Cooley 1993). The remaining proteins may represent further classes of POZ domain protein.

The POZ domain is usually located at the extreme amino terminus (see below). Conserved residues, the ma-

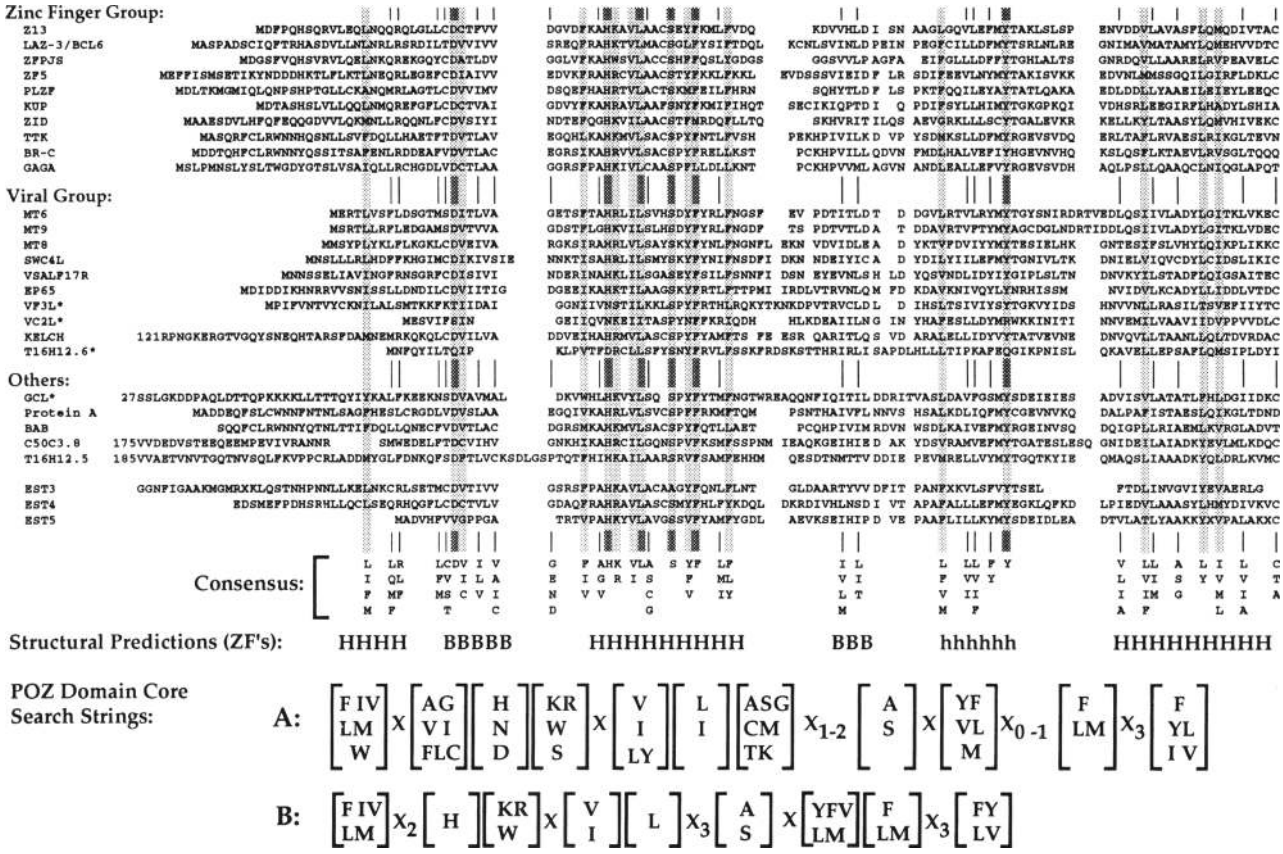

Figure 10. POZ domains. POZ domain sequences were aligned by the AMPS program (Barton and Sternberg 1987). Highly divergent domains are indicated (*). The consensus sequence is derived from positions where >85% of all POZ domains have the indicated amino acids. (*Bottom*) POZ domain motif search strings are shown. String *A* identifies all POZ proteins; string *B* all but the four more divergent proteins. Dark shading indicates positions with at least 85% identity; light shading for positions with only conservative amino acid substitutions. Structural predictions using a joint prediction program (E.E. Eliopoulos, University of Leeds, UK) for the 10 zinc finger POZ domains are shown. Regions scored by three or more algorithms in at least eight proteins are indicated by H (helix) and B (β sheet); (h) a helical region predicted in six of the nine proteins. Proteins and sequence accession numbers for the sequences are as follows: Z13, T. Schulz and J. Wells (pers. comm.); LAZ3/Bcl-6, Z21943 and U00115; ZFPJS, L16896; ZF5, L15325; PLZF, Z19002; KUP, X16576; Ttk X17121; BR-C X54663; GAGA, L22205; MT6, MT8, MT9 myxoma virus proteins T6, T8, T9 , M81919, M35233, M15806; SWC4L, swine poxvirus protein C4L, L22013; VSalF17R, VF3L, VC2L, vaccina virus proteins SalF17R, F3L, C2L, M35027; EP65, *Ectromelia* virus protein p65, Z14256; *kelch*, open reading frame 1, L08483; C50C30.8, T16H12.5 and T16H12.6, *C. elegans* putative proteins, L14433, Z30662; GCL, M97933; protein A, X75498; BAB, U01333; EST1-5, expressed sequence tags, M85329, Z19233, M85739, T07956, T08942. POZ proteins not included in Fig.: EST1 and EST2 do not encode intact domain; Shope fibroma virus proteins T6, T8, T9, M14899, M12332, M15921 are nearly identical to the myxoma proteins. While under review two more POZ domain genes were submitted to the data base: FBP, X79050 and Lola, U07607.

jority of which are hydrophobic, occur at 37 positions throughout the 120-amino-acid region; of these, 6 positions are identical in >85% of the sequences while at a further 11 positions only conservative substitutions are found. Additional positions show conservation among members of subfamilies such as the *Drosophila* zinc finger proteins or the viral proteins. Structural predictions for the POZ domains from the 10 zinc finger proteins suggest an alternation of helical and β-sheet structures; in the most highly conserved region a heptad repeat of hydrophobic residues is apparent. We have submitted to the Prosite motif data base two search strings that unequivocally identify the POZ family members in the current data base (Fig. 10).

The POZ domain mediates protein–protein interactions

Our data indicate that the ability to mediate protein interactions is a general property of POZ domains. Of the five POZ domains we have tested, those from ZID, ZF5, and the poxviruses exhibit only homomeric interactions, whereas those from the Ttk and GAGA proteins also participate in a heteromeric interaction. It should be possible to exploit the latter observation to map residues that determine the specificity of POZ domain interactions. The amino-terminal location of many POZ domains suggests that this potentially accessible location may be a prerequisite for interactions. However, several proteins contain sequences amino-terminal to the POZ domain, and the conservation of such sequences between the C50C3.8 and T16H12.5 proteins suggests that they are translated: Perhaps such proteins represent precursors containing a POZ domain that is exposed following proteolytic cleavage (see below).

We have not rigorously defined the stoichiometry of POZ domain interactions. Intact ZID protein generates heterogeneous complexes in the gel mobility retardation assay, which might result either from dissociation of multimers during electrophoresis or from unstable binding of dimers to two or more probe molecules. In contrast, simultaneous production of both intact ZID and a truncated derivative that retains the POZ domain but lacks the fingers, generates a heterodimeric, well-resolved DNA protein complex. Other data, however, are suggestive of multimeric interactions: The presence of the POZ domain allows the formation of large, uniform structures visible by electron microscopy (R. Newman, V. Bardwell, and R. Treisman, unpubl.), and sucrose velocity sedimentation experiments suggest that intact ZID and Ttk proteins are very large (V. Bardwell and R. Treisman, unpubl.).

An intact POZ domain appears necessary and sufficient for protein–protein interaction when tested in the coimmunoprecipitation assay or the DNA-binding "rescue" assay. In contrast, when efficiency of DNA binding was used to evaluate activity of the ZID amino-terminal sequences, deletion of either amino- or carboxy-terminal halves of the POZ domain failed to activate DNA binding. These observations suggest that subfragments of the POZ domain can exhibit residual interactions but that these must be weak compared with those of the intact domain.

Are POZ domain interactions regulated? A number of mechanisms for regulation can easily be envisaged. First, the POZ domain might be removed by specific proteolysis or by differential mRNA splicing (it should be noted, however, that of the various Ttk and BR-C mRNA isoforms identified, none encodes such a derivative (DiBello et al. 1991; Read and Manley 1992). Second, POZ domain interaction could be activated by proteolytic cleavage in which sequences amino-terminal to the POZ domain are removed. Third, POZ domain activity might be regulated by covalent modifications such as phosphorylation. Fourth, its interaction with other proteins either containing or lacking POZ domains might produce complexes with different functional properties. We are currently investigating these possibilities.

The POZ domain and DNA binding

We showed that in three zinc finger POZ proteins the presence of the POZ domain is associated with 5- to 10-fold reduction in DNA-binding affinity and the formation of heterogeneous complexes in the gel mobility retardation assay. Some DNA binding by the intact proteins is also detectable in immunoprecipitation, DNase I footprinting, and Southwestern blotting assays (V. Bardwell and R. Treisman, unpubl.). The inhibitory effect of the POZ domain does not require interaction with other cellular proteins and does not appear to be mediated by specific interactions between the POZ domain and the zinc finger region concerned. Most strikingly, the zinc finger region of ZID can be replaced by heterologous DNA-binding structures without compromising the inhibitory effect of the POZ domain. The simplest interpretation of our data is that in the dimeric or multimeric complexes, the protein–protein interactions mediated by the POZ domain effectively prevent the interaction of the associated DNA-binding domain with DNA.

Our results are in apparent contrast to several studies that demonstrate DNA binding by the Ttk, GAGA, and ZF5 proteins (Brown et al. 1991; Read and Manley 1992; Numoto et al. 1993; Soeller et al. 1993; Tsukiyama et al. 1994). However, these studies did not explicitly address the effect of the POZ domain on DNA binding; the activity reported may correspond to the weak binding we detect with the intact proteins. Alternatively, it is possible that the inhibitory effect of the POZ domain might have been relieved by limited proteolysis during purification, or by the addition of amino-terminal sequences to enable expression of recombinant fusion proteins. Another potential explaination is that POZ domain proteins purified from natural sources might be in a form in which they bind DNA efficiently.

Our observations raise the possibility that POZ zinc finger proteins do bind DNA efficiently but with a specificity different from the isolated finger domains. In a simple model, homo-oligomers of POZ proteins might bind novel DNA sequences not recognized by the iso-

lated finger regions. Our data do not support this idea. Although both the Ttk and ZF5 proteins have been reported to bind double targets containing two monomer sites separated by 29 and 16 bp, respectively (Brown et al. 1991; Numoto et al. 1993), intact Ttk does not efficiently bind such a target from the *ftz* promoter (V. Bardwell and R. Treisman, unpubl.). Moreover, binding-site selection experiments with intact Ttk and ZID recovered only conventional monomer consensus sequences from a pool of 26-bp random sequence oligonucleotides. Finally, while it is attractive to propose that POZ-mediated oligomerization of GAGA, which contains only one zinc finger, allows efficient DNA binding (Soeller et al. 1993), in our experiments this does not appear to be the case. However, it remains possible that heteromeric complexes between different POZ proteins do bind DNA, and this possibility will require further study.

Non-zinc finger POZ proteins

Our results suggest that non-zinc finger POZ proteins also utilize this domain as an oligomerization motif and that this will be reflected in their activity. For example, we found that the viral POZ domain could effectively substitute for that of ZID to inhibit DNA binding. The *Drosophila kelch* protein is localized in specialized structures of the intercellular bridges that connect the nurse cells of the developing oocyte (Xue and Cooley 1993); we speculate that oligomerization activity of the POZ domain is involved in this process. Perhaps the viral proteins, whose function is unknown but are apparently homologs of the *kelch* protein, form similar structures.

POZ domains and cancer

Two chromosomal translocations implicated in human cancer involve POZ domain zinc finger protein genes. One type of APL translocation fuses the POZ domain PLZF gene to that encoding the transcription factor RARα (Chen et al. 1993). This fusion protein is likely to be defective in DNA binding, as the ZID POZ domain inhibits DNA binding by many different types of DNA-binding domain, including that of RARα, when linked in *cis*. We suggest that the putative PLZF–RARα oncogene may act in a dominant-negative fashion by sequestering its partner RXR in an inactive multimeric complex. Consistent with this proposal, PLZF–RARα inhibits retinoic acid-induced transcriptional activation in transfection experiments (Chen et al. 1994). A similar hypothesis has been advanced in the case of other, more common, APL translocations, which join the non-POZ domain gene PML to the RARα gene (Grignani et al. 1994). Like the PLZF fusion, the PML–RARα resulting fusion contains a novel oligomerization domain, derived from PML, which both alters the DNA-binding properties of RARα (Perez et al. 1993) and causes the sequestration of RXR in large nuclear structures (Koken et al. 1994; Weis et al. 1994).

Up to 12% of non-Hodgkin's lymphomas are associated with translocations involving the 3q27 locus, which encodes the POZ zinc finger protein LAZ3/Bcl6 (Kerckaert et al. 1993; Ye et al. 1993; Miki et al. 1994); at least in one case, LAZ3 transcription is activated by its translocation into the IgH enhancer region (Kerckaert et al. 1993). A conventional model would suggest that this leads to inappropriate activation of LAZ3/Bcl6 target genes in lymphoid cells, in turn leading to transformation. However, in light of our results, it is reasonable to suggest an alternative model in which transformation arises by a dominant-negative mechanism involving the sequestration of partner POZ domain proteins. Both the PZLF and LAZ/Bcl6 translocations should provide good model systems for studying POZ protein activity in a biological context.

Materials and methods

Yeast reporter strains

A skeletal α-actin CArG box 1–*lacZ* reporter gene was constructed by insertion of four copies of the oligonucleotide 5′-tcgaCCCAACACCCAAATATGGCTCGAGAA-3′ into the *Xho*I site of pLGΔ-178 (pVB4; oligonucleotide orientation -+++). The reporter gene was embedded in the *Saccharomyces cerevisiae URA3*$^+$ coding region by insertion into the *Stu*I site of pURA3 (G. Micklem, unpubl.) creating pVB25, which was used to generate an indicator yeast strain S25 by disruption of the genomic *URA3* locus (Dalton and Treisman 1992).

Isolation of ZID cDNAs

S25 cells were transformed with a VP16 activator-tagged cDNA library (Dalton and Treisman 1992). Transformation was as described (Hill et al. 1991) with 1.5-μg carrier DNA/μg library DNA; filter sheets (Dalton and Treisman 1992) were transferred from Ura$^-$ to galactose medium after 24–28 hr. Approximately 5×10^5 transformants were screened by the colony color assay (Dalton and Treisman 1992) and plasmids were recovered from cells displaying a plasmid-dependent blue phenotype. Further ZID cDNAs were recovered from the VP16 cDNA library by polymerase chain reaction (PCR) using nested primers or by use of ZID nucleotides 465–808 as a probe for the screening of a λgt11 placental cDNA library.

Plasmids

Construction was by standard methods; all plasmid structures were verified by appropriate restriction digest or sequencing. For in vitro transcription/translation, coding sequences were into plasmid T7βplink or derivatives that place the 9E10 Myc epitope tag at the amino or carboxyl terminus (Dalton and Treisman 1992). Coding sequences were inserted into MLVplink (Dalton and Treisman 1992) or pEF–BOS (Mizushima and Nagata 1990), and into pET-21d (Novagen) for expression in mammalian cells and bacteria, respectively. Details of plasmid construction are available on request. The single-letter code, with 9E10 eptiope sequences underlined, is used throughout.

ZID derivatives ZID derivatives were inserted into T7plink or 9E10-tagged derivatives unless specified, ZID: [ZID codons 1–424 (complete protein)]. VΔ139Z: (MAGS)(VP16 codons 410–490)(ZID codons 139–424); also in MLVplink. tΔ139Z: (MEQKLISEEDLNMAGSEFPLCSKE)(ZID codons 139–424). Δ69Z: (M)(ZID codons 69–424). Δ122Z: (MAGS)(ZID codons 122–424). Δ139Z: (MAGSEFPLCSKE)(ZID codons 139–424).

Δ253Z: (MAGS)(ZID codons 253–424). Z69Δ139: (ZID codons 1–69)(EFPLCSKE)(ZID codons 139–424). Z122Δ253: (ZID codons 1–122 and 253–424). ZVt: (ZID codons 1–415)(VP16 codons 411–490)(EFLEQKLISEEDLN) (also in pEF–BOS). Δ122ZVt (MAGS)(ZID codons 122–415)(VP16 codons 411–490)(EFLEQKLISEEDLN) (also in pEF–BOS). ZID derivatives in pEF–BOS. Z212Δt (ZID codons 1–212)(AGSEFLEQKLISEEDLN). Δ122Z212Δt: (MAGS)(ZID codons 122–212)(AGSEFLEQKLISEEDLN). Z122Δ: (ZID codons 1–122)(SRSIRLV). Z212Δ: (ZID codons 1–212). ZH: (ZID codons 1–415)(DKLAAALEHHHHHH) in PET21d. Δ122ZH: (MAGS)(ZID codons 122–415)(DKLAAALEHHHHHH) (in PET21d) ZF5/Z: (ZF5 codons 1–126)(GSEFPLCSKE)(ZID codons 139–424). ZF5 sequences were generated by the PCR from mouse cDNA. Viral/Z: (Salf17R protein codons 1–110)(ZID codons 120–424). Z/SP1: (ZID codons 1–308)(Sp1 codons 530–696). ΔZ/SP1: (MAGS)(ZID codons 122–308)(Sp1 codons 530–696). Z/Oct-1: (ZID codons 1–308)(A)(human Oct-1 codons 270–442). ΔZ/Oct-1: (MAGS)(ZID codons 122–308)(A)(Oct-1 codons 270–442). Oct-1: (MAGS)(Oct-1 codons 270–440)(RSEFLEID). Z/SRF: (ZID codons 1–122)(SRSIRLVEASA)(SRF codons 1–244). SRF: (SRF codons 1–244).

Ttk derivatives Ttk derivatives were inserted into T7plink or 9E10-tagged derivatives. TTK: [p69Ttk codons 1–641 (complete protein)]. Δ153TTK: (M)(Ttk codons 153–641). TTKt: (Ttk codons 1–624)(EFLEQKLISEEDLN). Δ153TTKt: (M)(Ttk codons 153–624)(EFLEQKLISEEDLN). TTK153Δt: (Ttk codons 1–153)(ASEFLEQKLISEEDLN).

GAGA derivatives GAGA cDNAs were generated from 4 to 12-hr *Drosophila* embryo cDNAs by PCR using high-fidelity polymerase and inserted into T7plink. GAGA: [GAGA codons 1–519 (complete protein)]. Δ280GAGA: (GAGA codons 280–519).

RAR derivatives and RXR RARα derivatives were inserted into T7plink. Complete human RARα and RXR were synthesized from pSG5–RARα and pSKXR3-1 respectively. Z/RAR: (ZID codons 1–212)(RAR α codons 20–462). ΔZ/RAR: (ZID codons 122–212)(RAR α codons 20–462). RAR: (RAR α codons 1–462). RXR: (RXR codons 1–462).

CAT reporter plasmids

Double-stranded oligonucleotides of a ZID consensus binding site (5′-CTAGTGGCTCCATCATC-3′) or a mutated one (5′-CTAGTGGGTGCATCATC-3′) were inserted into the *Xba*I site of pBLCATΔBam (Hill et al. 1993).

Cell culture, transfections, and immunofluorescence

NIH-3T3 cell maintenance, transfection, and extract preparation were as described previously (Hill et al. 1993); cells were harvested after 40 hr incubation in medium containing 10% fetal calf serum. For immunofluorescence transfected cells on coverslips were fixed in 4% paraformaldehyde (pH 7.3), blocked with 3 mg/ml of immunoglobulin-free BSA, 1% Triton X-100, in PBSA. Antibody incubations (30–60 min) were in PBSA with 3 mg/ml of BSA and 0.1% Triton X-100: (1) 9E10 (1:1000); (2) rabbit anti-mouse (1:100); (3) fluorescein-conjugated swine anti-rabbit (1:100). Washes were in PBSA, 0.1% Triton X-100.

DNA-binding site probes

Oligonucleotides containing ZID, Ttk, SP1, Oct-1, and SRF sites were inserted into plasmid polylinkers and probes generated by PCR using appropriate primers. RAR/RXR probe (DR+5) was generated by labeling overlapping complimentary oligonucleotides. Protein-binding sites are underlined and linker sequences are in lowercase. TTK: 5′-CATTGTTAATGGACGTTATCCTTATTAGATGTTGATGTCCCA-3′, from the *fushi tarazu* enhancer 1610–1651 bp (Harrison and Travers 1990). GAGA: 5′-CTCGCCCTCTCGCTCCCcgcagct3′, site C from the *engrailed* promoter 2301–2317 bp (Soeller et al. 1993). SP1: 5′-AATTCCCAGAGCCCCGCCCCCGGCTCCAGGATC-3′, a synthetic site. Oct1: 5′-GATCCATGCAAATGAAGATC-3′ , a synthetic site. SRF: 5′-ctagAGGATGTCCATATTAGGACATCT-3′, the c-*fos* serum response element (Hill et al. 1993). DR+5 5′-CTAGTCTATGAGGTCACCAGGAGGTCAAACTTC-3′, a synthetic site.

Immunoprecipitation assays

Reactions (50 μl, containing 5 μl of reticulocyte lysate and 200 ng of 9E10 antibody) were set up as for DNA binding, but without DNA. Ten microliters of a 50% slurry of protein A–Sepharose (prebound with rabbit anti-mouse antibody in reaction buffer) was added and, after incubation for 1–2 hr at 4°C with rotation, the beads were recovered by centrifugation and washed twice with 0.5 ml of cold RIPA buffer (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 0.5% deoxycholate, 0.5% NP-40, 0.05% SDS, 10% glycerol, 10 mM EGTA, and protease inhibitors). Bound proteins were eluted by boiling beads in 2× SDS–gel loading buffer and fractionated on 15% SDS–polyacrylamide gels.

Expression of ZID in E. coli

Expression and purification of histidine-tagged recombinant proteins was carried out using Novagen protocols. Purified proteins were dialyzed against 100 volumes of 50% buffer D0.1 (Marais et al. 1993) containing 0.5 mM $ZnCl_2$, 1 mM DTT, and 4 M urea for 1 hr, against the same buffer with 2 M urea (1 hr), then no urea (1 hr), and again overnight without urea. Protein concentration was estimated by Coomassie staining after SDS-PAGE.

Other methods

In vitro transcription was as described previously; for cell-free translation, rabbit reticulocyte lysate (Promega) was used according to the manufacturer's instructions and included 50 μM $ZnCl_2$. Gel mobility retardation assays were carried out as described (Pollock and Treisman 1990; Marais et al. 1993) using 0.1 mg/ml of poly[d(I-C)] (0.05 mg/ml for RAR/RXR), 2.5 or 5 fmoles of probe and 0.75–1.5 μl of reticulocyte lysate or 1 ng of purified protein. Equal amounts of proteins and lysate were used in any given experiment. For whole cell extracts from transfected cells, 15 μg was used. Gel mobility retardation assays with GAGA were carried out at 4°C. Site selections and DNA coimmunoprecipitations were carried out essentially as described previously (Pollock and Treisman 1990, 1991). The PhosphorImager was used for quantitation where necessary. Sequencing, SDS-PAGE, and immunoblotting with detection by enhanced chemiluminescence assay were carried out by standard methods.

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