Are many Z-DNA binding proteins actually phospholipid-binding proteins? (porins/annexins/lipoproteins/gene regulation/recombination)

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Communicated by Gary Felsenfeld, December 4, 1989 (received for review July 26, 1989)

ABSTRACT We used a Z-DNA affinity column to isolate a collection of Z-DNA binding proteins from a high salt extract of Escherichia coli. We identified one of the major Z-DNA binding proteins of this fraction, not as a protein involved in gene regulation or genetic recombination, but rather as an outer membrane porin protein. We then showed that several other known phospholipid-binding proteins (bovine lung annexins and human serum lipoproteins) also bind much more tightly to Z-DNA than to B-DNA. In all cases, this Z-DNA binding was strongly blocked by competition with acidic phospholipids, such as cardiolipin. Our results raise the question whether many of the Z-DNA binding proteins previously isolated are actually phospholipid-binding proteins.

The biological significance of left-handed Z-DNA remains unknown. Although appropriate regions of natural DNA can be made to adopt a left-handed conformation in vitro (1-5), which can then influence a number of biochemical processes (6-11), most evidence shows that similar effects do not occur in vivo (10-13). On the other hand, proteins that clearly prefer to bind to Z-DNA rather than to B-DNA have been isolated from a number of natural sources (14-23). Although the identity and functions of these proteins are largely unknown, the existence of such proteins has been taken as evidence for a biological function of Z-DNA.

In the present paper, we consider whether the existence of Z-DNA binding proteins indeed provides support for the natural existence of Z-DNA. Our concerns arise because of our recent and quite unexpected finding that yolk proteins from nematodes, chickens, and frogs bind strongly and preferentially to Z-DNA (24). Although yolk proteins have a higher Z-DNA specificity than most of the Z-DNA binding proteins previously isolated, it is unlikely that they bind to Z-DNA in vivo. Thus, if we had not happened to identify these proteins, they would have been accepted as bona fide Z-DNA binding proteins, with some yet to be defined biological function. We thus became curious about the true identity of Z-DNA binding proteins isolated purely by physical criteria. In particular, since yolk proteins appear to bind Z-DNA at a phospholipid-binding site (24), we became suspicious that at least some of the previously isolated Z-DNA binding proteins might actually be phospholipid-binding proteins.

In the present paper, we used the Z-DNA affinity column developed by Rich and coworkers (14, 16, 17, 21, 23) to isolate Z-DNA binding proteins from a high salt extract of Escherichia coli. We identified one of the most prominent of these proteins as an outer membrane porin protein, which is unlikely to be involved in either gene regulation or genetic recombination. We then show that phospholipid-binding proteins as diverse as bovine lung annexins and human serum lipoproteins all bind strongly and preferentially to Z-DNA. We feel that our results raise the distinct possibility that many of the uncharacterized Z-DNA binding proteins previously isolated are actually phospholipid-binding proteins.

MATERIALS AND METHODS

Nucleic Acids. The synthesis of labeled and unlabeled poly(dG-dC), its conversion to the Z-DNA conformation, and verification of the latter structure were carried out as described (24).

Preparation of Z-DNA and B-DNA Affinity Matrix. For preparation of the Z-DNA and B-DNA affinity matrix, 120-130 A260 units of brominated poly(dG-dC) or 120 A260 units of poly(dG-dC), respectively, were covalently coupled to 250 mg of G-200 (particle size, 40-120 μm; Pharmacia) by using 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-p-toluene sulphonate as the coupling agent (25).

Preparation of E. coli Extract. Bacteria (E. coli, strain 08:K27) were grown in M-9 medium (26) to an A600 of 0.8, centrifuged (4000 x g for 30 min), washed once in a buffer containing 10 mM Tris-HCl (pH 8.0), 1 M NaCl, and 1 mM EDTA, and resuspended in sonication buffer [50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.2% Triton X-100]. The cells were broken by sonication at a power setting of 50 W for a period of 1-2 min per ml of extract, the extract was diluted to a concentration of 0.5 M NaCl, and MgCl2 and Dnase I were added to a concentration of 10 mM and 10 μg/ml, respectively (27). The extract was digested for 30 min at 10°C. EDTA was then added to 2 mM, the extract was centrifuged at 20,000 x g for 30 min at 4°C, and the supernatant was dialyzed extensively against the binding buffer (100 mM NaCl/10 mM Tris-HCl, pH 8.0/1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/1 mM dithiothreitol). To 3 mg of this protein extract, 3 mg of sonicated native E. coli DNA was added, and the mixture was again dialyzed against binding buffer, centrifuged at 20,000 x g for 30 min at 4°C, and used for affinity chromatography.

DNA Affinity Chromatography. The columns were equilibrated in the binding buffer at 4°C. The high salt E. coli extract containing competitor DNA (see above) was applied to each column, which was then washed extensively with the binding buffer, and finally eluted by the addition of 1 M NaCl in binding buffer. The eluate was dialyzed against 20 mM NH4HCO3, lyophilized, and analyzed on a SDS/7.5-15% polyacrylamide gradient gel.

Proteins. Porins were purified from E. coli (strain 08:K27) essentially as described by Nakaido (28). Annexins were purified by the procedure of Khanna et al. (29). Plasma lipoproteins were obtained from Sigma (catalogue nos. L-

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein.

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Nitrocellulose Filter Binding Assays. Nitrocellulose filter binding experiments were carried out as described (24). Typical binding reaction mixtures (100 µl) contained 10 mM Tris·HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.25 µg of the [3H]DNA probe, and various amounts of proteins. The mixture was incubated for 30 min at 37°C, applied to a nitrocellulose filter (Millipore type HA; 0.45 µm, prewashed in 0.3 M NaOH), and washed with 2 ml of the binding buffer. The filters were dried and retained radioactivity was measured by liquid scintillation counting. In competitive filter binding assays, proteins were preincubated for 10 min with the competitor, the Z-DNA probe was then added, and the reaction was incubated for a further 30 min at 37°C before filtration. As previously (24), we verified that phospholipids did not prevent the various proteins from binding to the nitrocellulose filter. The filter binding assays for the annexin class of proteins were performed in a buffer (binding and washing) containing 10 mM Tris·HCl (pH 7.5), 100 mM NaCl, 0.1 mM EGTA, and CaCl₂ concentrations as indicated.

RESULTS AND DISCUSSION

Z-DNA Binding Proteins in E. coli. Our original aims were to purify and identify a Z-DNA binding protein from E. coli. Lafer et al. (23) had previously isolated a number of such proteins by passing low salt extracts of E. coli over a Z-DNA affinity column in the presence of excess B-DNA. Our procedure was similar to that described by Lafer et al. (23), with one major difference: to ensure complete extraction of chromosomally bound proteins, we increased the salt concentration in our extraction buffer to 0.5 M NaCl (27).

High salt extracts of E. coli were mixed with native E. coli DNA (1 mg of DNA per mg of protein) and passed over a Z-DNA affinity column. After the column was washed extensively with 0.1 M NaCl, bound proteins were eluted with 1 M NaCl and displayed on the SDS/polyacrylamide gel shown in Fig. 1A (lane A). As a control, the same extracts were passed over a B-DNA column and the 1 M NaCl eluted proteins are shown in lane B. The gel profile of the total extract loaded onto the columns is shown in Fig. 1B.

It is clear from Fig. 1 that, as expected, only a few of the many hundreds of E. coli proteins bind either to Z-DNA or to B-DNA columns. However, we were somewhat perturbed that there appeared to be a greater number of Z-DNA binding proteins than B-DNA binding proteins; this would not be expected if Z-DNA binding proteins participated in only a subset of gene control processes. We were also perturbed by the fact that the detailed distribution of Z-DNA binding proteins depended on E. coli strain, on growth conditions, and on extraction procedure (not shown). Nonetheless, the major proteins of the Z-DNA binding fraction were detected under a variety of conditions, and we judged the column profile to be sufficiently reproducible to proceed with protein purification.

Lafer et al. (23) had reported that the major Z-DNA binding proteins in E. coli had molecular masses of 50, 90, and 100 kDa. In some, but not all, of our isolations, we too could identify Z-DNA binding proteins of roughly this size. However, in all of our extracts, the most prominent Z-DNA binding protein has a molecular mass of 36 kDa (marked in Fig. 1). The fact that this 36-kDa protein is enriched in high salt extracts compared to the low salt extracts of Lafer et al. (23) originally suggested to us that it might actually be bound to the E. coli chromosome in vivo and made it appear as an attractive candidate for characterization.

Is the 36-kDa Z-DNA Binding Protein of E. coli Actually a Membrane Protein? By the usually accepted physical criteria, the 36-kDa protein certainly qualifies as a Z-DNA binding protein. However, we were reluctant to conclude that the protein therefore binds to Z-DNA in vivo. We were suspicious for two reasons: (i) as shown in Fig. 1, the 36-kDa protein can be aligned with a major protein in the total extract; such prevalence is rather unexpected for a protein involved in gene regulation; and (ii) yolk proteins, judged by the same physical criteria, are among the most avid and most specific Z-DNA binding proteins yet identified (24).

The prevalence of the 36-kDa protein, coupled with our suspicion that phospholipid binding sites might actually be involved in Z-DNA binding (24), suggested that we could possibly identify this protein with a known E. coli protein. One of the porin proteins looked like the obvious candidate because (i) most porins have molecular masses in the range of 35 to 38 kDa; (ii) they are a major cell protein, present in 10⁷–10⁸ copies per E. coli cell; (iii) they are solubilized only under high salt conditions; and (iv) they are normally situated in the E. coli outer membrane and interact with phospholipids (31, 32).

We thus purified porins by well established methods (28). The nitrocellulose filter binding assay shown in Fig. 2 demonstrates that purified porins do indeed bind to Z-DNA.
much more avidly than they bind to B-DNA. We estimate the minimum association constant of porins to Z-DNA to be on the order of 10^4 M^-1. This is certain to be an underestimate, because of the method of analysis (24), because not all porin species could be active in binding, and, probably most importantly, because of unknown quantities of phospholipid associated with the purified porins. As described below, phospholipids are potent inhibitors of Z-DNA binding.

The data of Fig. 2, along with competition experiments shown below, suggest that the porin Z-DNA specificity (defined as the ratio of the intrinsic association constants to Z-DNA and to B-DNA) is at least 50-fold and probably much higher. Thus, porins are more "Z-DNA specific" than most previously investigated Z-DNA binding proteins (see comparisons in ref. 24).

We thus conclude that one of the major Z-DNA binding proteins of E. coli is really an outer membrane porin protein. Furthermore, it is unlikely that this protein has anything to do with Z-DNA in vivo.

Other Phospholipid-Binding Proteins Interact Strongly and Preferentially with Z-DNA. The above results raise the possibility that other (perhaps all) phospholipid-binding proteins also bind preferentially to Z-DNA. To determine whether it is indeed the phospholipid-binding characteristics of a protein that are involved in Z-DNA binding, we looked for a situation in which the phospholipid-binding of a protein could be altered experimentally. We could then observe whether any Z-DNA binding properties were likewise altered.

The annexins (which include calpactins and lipocortins) are a class of eukaryotic proteins that seem ideally suited for this experiment, since they bind phospholipid in a Ca^{2+}-dependent manner (reviewed in refs. 33-35). We thus purified annexins from bovine lung (29) and assayed for their Ca^{2+}-dependent binding to Z-DNA. Fig. 3 A and B shows that annexins do indeed bind to Z-DNA much more avidly than they bind to B-DNA. Moreover, the binding to Z-DNA is strongly Ca^{2+}-dependent.

The annexins, as isolated from bovine lung, are a group of about nine different proteins, and a Western blot probed with Z-DNA reveals that several protein species contribute to the Z-DNA binding (data not shown). To be certain that our conclusions are not somehow confounded by protein heterogeneity, we repeated the filter binding experiments on a single purified annexin termed p70. Fig. 3C shows that homogenous p70 also binds Z-DNA in a Ca^{2+}-dependent manner. In both the presence and the absence of Ca^{2+}, p70 strongly prefers Z-DNA over B-DNA.

While we had no intention of screening the Z-DNA binding properties of all known phospholipid-binding proteins, we were nonetheless interested in determining whether the association between the two binding activities was a general phenomenon. As a possibly extreme example, we consider two classes of lipoproteins found in human serum, the high density lipoproteins (HDLs) and the low density lipoproteins (LDLs), both of which are known to bind substantial quantities of phospholipids in vivo (36). As shown in Fig. 3D, both HDLs and LDLs also bind Z-DNA in preference to B-DNA.

We could detect no Z-DNA binding with purified phospholipase A2 or phospholipase C. However, proteins that interact enzymatically with phospholipids might be expected to behave differently from proteins that interact physically with bulk phospholipids, as, for example, in cell membranes.

Z-DNA Binding Can Be Inhibited by Phospholipids. Fig. 4 shows typical data demonstrating that the Z-DNA binding of HDLs, LDLs, and total E. coli proteins can be completely inhibited by modest amounts of the phospholipid cardiolipin. However, the same Z-DNA binding is weakly blocked by even large excesses of B-DNA, either E. coli DNA (Fig. 4) or the brominated polynucleotide poly[d(A-BR)] (data not shown). Equivalent results (not shown) are obtained with porins and annexins. Phosphatidyglycerol also inhibits Z-DNA binding but less efficiently than cardiolipin. It is difficult to analyze the inhibition curves quantitatively because of the unknown quantities of phospholipid associated with the purified proteins. Nonetheless, a minimal conclusion is that...
cardiolipin is at least a 50-fold better competitor of the Z-DNA binding than is an equivalent mass of B-DNA.

By and large, the above results are consistent with the simple notion that all Z-DNA binding proteins are phospholipid-binding proteins and vice versa. However, there are exceptions to this rule and it is difficult to be either rigorous or comprehensive. For example, phospholipids do not inhibit the Z-DNA binding of our polyclonal anti-Z-DNA antiserum (24), whereas they do inhibit the binding of lac repressor to its (B-form) operator sequence (data not shown). Thus, it is difficult to argue in general that phospholipid inhibition of DNA binding is diagnostic of artificial interactions. Nonetheless, the data show that there is no Z-DNA binding activity in E. coli extracts that cannot be inhibited by cardiolipin.

SUMMARY AND CONCLUSIONS

A considerable number of Z-DNA binding proteins from a variety of biological sources have now been identified by physical criteria. The expectation generated by these studies is that such proteins interact in vivo with naturally occurring regions of Z-DNA and that they participate in processes such as gene regulation or genetic recombination. The results presented here show that such physical tests of preferential binding to Z-DNA may not be sufficient to eliminate the prominent class of phospholipid-binding proteins. This raises the possibility that many of the previously identified Z-DNA binding proteins could have little to do with Z-DNA binding in vivo. We note that some Z-DNA binding proteins are found in the cytoplasm; although this has been attributed to nuclear leakage, it is also possible that these proteins are in fact normally associated with cell membranes, rather than with DNA. In those cases in which Z-DNA binding proteins have been isolated from nuclei, it has not, to our knowledge, been demonstrated that the same proteins are confined to nuclei and absent elsewhere in the cell. Such a demonstration now seems particularly important in distinguishing functionally significant Z-DNA binding proteins from those that are not.

It is both possible and tempting to invent schemes in which the binding of Z-DNA to lipoproteins actually reflects a physiological interaction. However, it is our judgment that such schemes are improbable, and it seems more likely that the interactions we describe here are completely adventitious. In the case of the yolk proteins, the structure of the phospholipid-binding cavity (30) suggested how it might also interact with Z-DNA (24). It remains to be seen whether the Z-DNA binding property of other phospholipid-binding proteins has a similar explanation.

These results do not mean that there is no such thing as a protein with a biological function that involves binding to Z-DNA. However, our data show that the simple demonstration of even strong preferential binding to Z-DNA, although necessary, is not sufficient as proof of the functional significance of the interaction.

The authors wish to thank T. Cyron and D. Hunt for preparation of the manuscript. This work was supported by the Medical Research Council of Canada and by the Alberta Heritage Foundation for Medical Research.