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Structure–function relationships in a bacterial DING protein

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Abstract A recombinant DING protein from *Pseudomonas fluorescens* has been previously shown to have a phosphate-binding site, and to be mitogenic for human cells. Here we report the three-dimensional structure of the protein, confirming a close similarity to the "Venus flytrap" structure seen in other human and bacterial phosphate-binding proteins. Site-directed mutagenesis confirms the role of a key residue involved in phosphate binding, and that the mitogenic activity is not dependent on this property. Deletion of one of the two hinged domains that constitute the Venus flytrap also eliminates phosphate binding whilst enhancing mitogenic activity.

Keywords: DING protein; Phosphate binding; Human phosphate-binding protein; pstS protein

1. Introduction

DING proteins were identified in a wide range of eukaryotic organisms, on the basis of conserved DINGGG N-terminal amino acid sequences [1,2]. Despite much effort, no complete gene sequences are known for these proteins, and only a single complete amino acid sequence. The latter was determined from the X-ray crystallographic structure determination of a human phosphate-binding protein (HPBP), purified from the high-density lipoprotein fraction of plasma, and confirmed by mass spectrometric peptide sequencing [3–6].

The tertiary structure of HPBP confirms the ability of this protein to bind a single phosphate ion, in the same manner as the bacterial pstS proteins, which sequester phosphate for cellular uptake by the ABC phosphate transporter. The pstS and the HPBP structures fit a model known as the ''Venus flytrap'', in which two globular domains hinge together to form the phosphate-binding site, with eight conserved residues H-bonding to phosphate [5,7,8].

Partial gene and protein sequences are also known for several plant DING proteins [1,2], one of which was isolated and clon\ed from St. John's Wort (*Hypericum perforatum*) on the basis of anti-HIV activity in lymphocytes. This $p27^{SJ}$ protein is clearly homologous to existing DING sequences [2], but is C-terminally truncated, possibly as a result of proteolysis by coagulation factor Xa action during the preparation of plant extracts [9]. In the $p27^{SJ}$ protein, all of the phosphate-binding residues are conserved, but it is not known if the protein binds a phosphate ion.

PstS proteins are ubiquitous in bacteria, but some *Pseudomonas* genomes contain genes which are much more homologous to the eukaryotic DING proteins. These genes form two families of phosphate-binding proteins, which may be involved in phosphate scavenging [2]. One such gene, from *P. fluorescens*, has been cloned and expressed [10]. The resulting protein (PfluDING) has a single phosphate-binding site. It also possesses mitogenic activity towards human fibroblasts, which is consistent with similar activity ascribed to human DING proteins [11–14]. In stimulating DNA synthesis, the bacterial DING protein does not stimulate cellular phosphate uptake [10]. The PfluDING sequence most closely resembles that of $p27^{SJ}$ (Fig. 1).

Because there are no complete DING gene sequences from eukaryotes, PfluDING offers the most convenient model for pursuing structure–function relationships in DING proteins. We here describe experiments to establish the tertiary structure of the PfluDING protein, and to investigate further the phosphate-binding role in relation to mitogenic activity.

2. Methods

A recombinant PfluDING protein was expressed in *E. coli* BL21 (DE3) as a fusion protein with a C-terminal hexahistidine tag, coded in the pET22b(+) plasmid vector, and purified from bacterial lysates by affinity chromatography, as previously described [10].

Crystallization of PfluDING was performed using the hanging drop vapor diffusion method and by mixing the reservoir solution composed of 25% (w/v) PEG 8000, 100 mM acetate buffer pH 4.5 and 200 mM Li_2SO_4 with a PfluDING solution at 10 mg/mL. A single dataset was collected at the BM14 UK MAD beamline (ESRF, Grenoble, France) at a wavelength of 0.953 Å. Crystallization and data collection are further described in Moniot et al., 2007 [15]. Statistics of the dataset are summarized in Table 1.

The initial phase was obtained using the molecular replacement method with the MOLREP program [16]. Since the PfluDING amino acid sequence displays 71% identity with the HPBP sequence, HPBP structure was used as a starting model (PDB code 2CAP). The PfluDING model was built through refinement and manual building cycles using REFMAC5 [17] and Coot [18]. The final model shows R and R_{free} factor values of 13.1% and 16.4%, respectively (Table 1). Coordinates and associate structure factor files have been deposited within the Protein Data Bank (ID code 2Q9T). Figs. 2–5 were drawn with PyMOL (http://www.pymol.org).

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PfluDING    DINGGGATLPQALYQTSGVLTAGFAQYIGVGSGNGKAAFLNNDYTKFQAGVTNKNVHWAGSDSKLSA
HPBP        DINGGGATLPQKLYLTPDVLTAGFAPYIGVGSGKGKIAFLENKYNQFG-TDTTKNVHWAGSDSKLTA
HypDING   MADINGGGATLPQALYQTSGVLTAGFAPYIGVGSGNGKAAFLNNDYTKFQAGVTNKNVHWAGSDSKLSA

PfluDING    TELSTYASAKQPTWGKLIQVPSVGTSVAIPFNKSGSAAVDLSVQELCGVFSGRINTWDGISGSGRTG
HPBP        TELATYAADKEPGWGKLIQVPSVATSVAIPFRKAGANAVDLSVKELCGVFSGRIADWSGITGAGRSG
HypDING     TELSTYASAKQPTWGKLIQVPSVGTAVAIPFNKSGTAAVDLSVSELCGVFSGRITDWSGISGSGRTG

PfluDING    PIVVVYRSESSGTTELFTRFLNAKCNAETGNFAVTTTFGTSFSGGL--PAGAVAATGSQGVMTALAA
HPBP        PIQVVYRAESSGTTELFTRFLNAKCTTQPGTFAVTTVFANSYSLGLSPLAGAVAAIGSVGVMAADND
HypDING     AITVVYRSESSGTTELFTRFLNAKC-AETGTFNISTTFGTSYTGGL--PAGAVSAAGSQGVMTALAG

PfluDING    G---DGRITYMSPDFAAPTLAGLDDATKVARVGKNVA--TNTQGVSPAAANVSAAIGAVPVPAAADR
HPBP        VTTAQGRITYISPDFAAPTLAGLDDATKVARTGKGSSSGGGAEGKSPAAANVSAAISVVPLPAAADR
HypDING     A---DGGTTYMSPDFAAPTLAGLDDATKVARVGKDVA--TNTAGVSPAAANVSAAINAVPVPASTEK

PfluDING    SNPDAWVPVFGPDNTAGVQPYPTSGYPILGFTNLIFSQCYADATQTTQVRDFFTKHYGASNNNDAAI
HPBP        GDPNVWTPVFGAVTGGGVVAYPDSGYPILGFTDLIFSECYANATQTGQVRNFFTKHYGTSANDNAAI
HypDING     P-----------------------------------------------------------------

PfluDING    TANAFVPLPTAWKATVRASFLTASNALSIGNTNVCNGIGRPLLE
HPBP        QANAFVPLPSNWKAAVRASYLTASNALSIGDSAVCGGKGRPE
HypDING     ----------------------------------------
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Fig. 1. Alignment of the amino acid sequences of three DING proteins. PfluDING: the recombinant PfluDING protein sequence. HPBP: human plasma phosphate-binding protein sequence. HypDING: *Hypericum perforatum* DING protein sequence, p27^{SJ}. Matches in blue, with the conserved residues involved in phosphate binding bold and underlined. Sequences are aligned with reference to the structural superposition of the PfluDING and HPBP ternary structures.

Table 1
Dataset and refinement statistics

Data collection	
Wavelength (Å)	0.9535
Resolution (last bin) (Å)	62–1.43 (1.50–1.43)
Space group	$P2_1$
Unit cell (Å)	$a = 36.7$, $b = 123.7$, $c = 40.8$, $\alpha = 90.0$, $\beta = 116.7$, $\gamma = 90.0$
Observed reflections (last bin)	235 730 (21 073)
Unique reflection (last bin)	58 905 (7166)
Completeness (last bin) (%)	98.1 (89.2)
R_{sym} (last bin) (%)	2.6 (10.2)
Mean $I/\sigma(I)$ (last bin)	38.9 (10.5)
Refinement statistics	
Resolution range (Å)	61.90–1.43
R_{work}/R_{free}	13.1/16.4
Number of protein atoms	3743
Number of water molecules	766
Average B factor (Å^2)	10.15
Rms bond length (Å)	0.007
Rms bond angle (°)	1.119

$R_{work} = \frac{\sum F_o - F_c}{\sum F_o}$, where F_o denotes the observed structure factor amplitude and F_c denotes the structure factor amplitude calculated from the model, R_{free} is as for R_{work}, but it is calculated with 5% of randomly chosen reflections omitted from the refinement.

Prior to mutagenesis, possible structural changes in putative variants were explored, using the Psipred software [19]. A mutated protein with a T147N substitution was created by overlap extension PCR [20] with PfluDING cDNA as the starting template, using the following primers (forward SAD1: 5′GC GAA AGC AGT GGT AAC ACT GAG CTG TTC, reverse SAD2: 5′GAA CAG CTC AGT GTT ACC ACT GCT TTC GC) in conjunction with the forward and reverse primers originally used to amplify the PfluDING coding sequence (MD5 and MD6), and using the same conditions for the PCR reaction [10]. Following a sequence check of the mutated cDNA, restriction, ligation and cloning into the pEt22b+ plasmid were carried out as before [10].

To create the truncated version of PfluDING, the primers NT1 (5′ CAA CCA TGG TGG GGA CTT CGG TTG CC) and CT2 (5′ CGT CTC GAG TGC GCC GAT AGC GGC AGA CAC) were created, and used for PCR with the normal PfluDING cDNA as template. NT1 causes truncation immediately before the codon CTG, corresponding to V90 in PfluDING, and CT2 caused truncation immediately after the codon GCA, for A252. The resulting cDNA was of about 500 bps, corresponding to the predicted size of 506 bps, and had the correct nucleotide sequence. The primers contained restriction sites for NcoI and XhoI, respectively, so the cDNA was restricted, ligated and cloned into the pET22b(+) plasmid, as for other PfluDING preparations. Both mutated PfluDING derivatives were expressed and purified as for the normal protein.

Western blotting was carried out with a rabbit antiserum to a conjugated human DING protein N-terminal peptide, as previously described [13]. Double immunodiffusion was carried out with a rabbit antiserum prepared against purified recombinant PfluDING, using standard immunization protocols.

Filter-binding assays for ^{32}P-phosphate binding to DING proteins, and mitogenic assays, based on incorporation of ^{3}H-thymidine, were also carried out as before [10]. For the phosphate-binding experiments, 3.6 μg of each protein was used, in triplicate assays. Differing concentrations of each protein (0.6–3.0 μM) were added to cell cultures for mitogenic assays.

3. Results and discussion

The three-dimensional model of PfluDING reveals an elongated fold composed of two adjacent globular domains. Each domain is constituted by a central β-sheet core flanked by α-helices and contains a disulfide bridge (C114–C159 and C300–C363). Interconnected by an antiparallel two-stranded β-sheet acting as a hinge, the two domains form a deep cleft wherein is bound a phosphate molecule. This fold, known as a Venus flytrap, is very similar to those of the sixth family of solute binding proteins (SBP) [21,22]. Structural superposition (Fig. 2) shows good correspondence between our model and the structures of *E. coli* phosphate-binding protein (pdb code 1IXH) and HPBP (pdb code 2CAP), with root mean square deviations (rmsd) of 1.881 Å on 276 Cα atoms and 0.649 Å on 366 Cα atoms, respectively. Similar to the eukaryotic HPBP [5], the PfluDING structure exhibits four protruding loops when compared with the *E. coli* binding protein. Though these loops are a little shorter, they superimpose almost perfectly

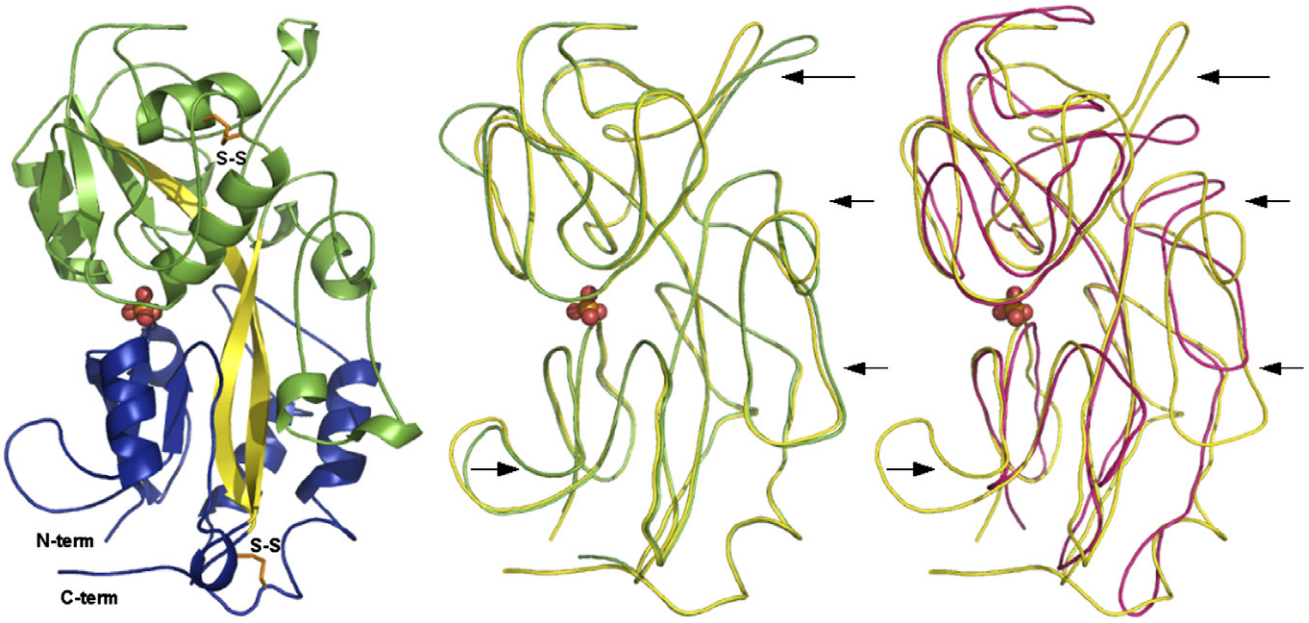

Fig. 2. Ternary structure superposition. (*left*) The overall structure of the DING protein from *Pseudomonas fluorescens* is shown in cartoon mode. The two domains of the protein are colored in blue and green and the hinge is presented in yellow. Both disulfide bridges are presented in orange. The phosphate molecule is represented in ball and stick mode (in red). (*middle*) Structural comparison between the *P. fluorescens* DING protein (in yellow) and the human phosphate-binding protein (HPBP) (pdb code 2CAP) (in green). (*right*) Structural comparison between the *P. fluorescens* DING protein (in yellow) and the phosphate-binding protein from *Escherichia coli* (pdb code 1IXH) (in purple). The phosphate from the PfluDING structure is represented in ball and stick mode (in red). Protruding loops of PfluDING and HPBP are indicated by arrows.

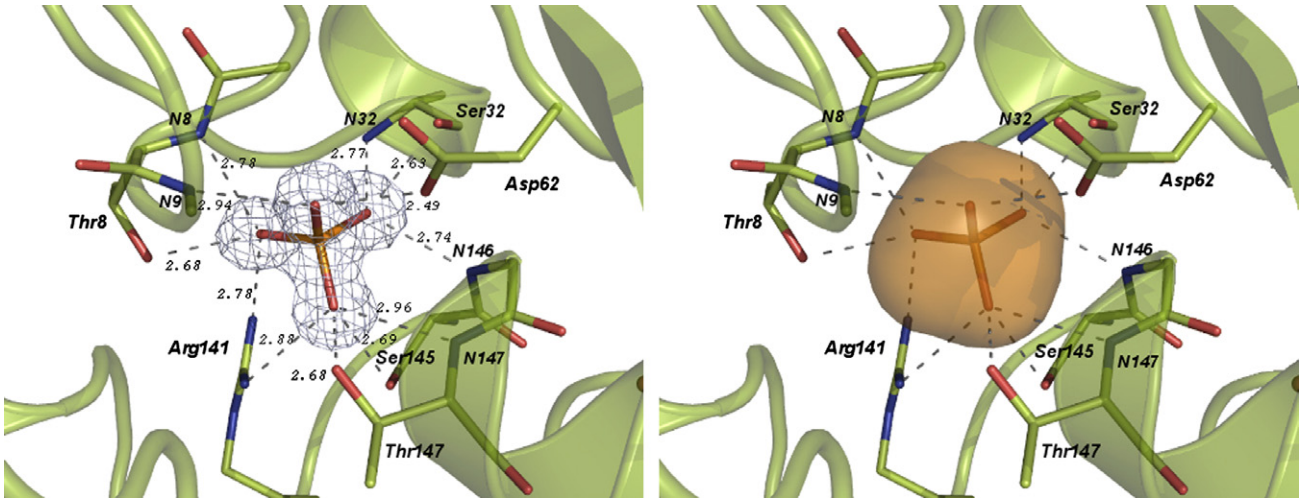

Fig. 3. Views of the phosphate-binding site. Residues involved in the binding of the phosphate molecule are labeled. The 12 H-bonds between the protein and the phosphate molecule are represented in dashed lines. Side chains are shown in sticks and main chain is represented in cartoon mode. (A) The $2F_o - F_c$ map calculated omitting the phosphate molecule was contoured at 5σ. Hydrogen bond distances are indicated in Å. (B) Binding site cavity. Surface is compute from the protein Van der Waals surface using a 1.6 Å probe sphere radius.

with those of HPBP (Fig. 2). Structural comparison thus reveals that PfluDING superimposes more closely on HPBP, and possesses similar protruding loops. This strongly supports the hypothesis deduced from the high sequence identity between both proteins (71%) that bacterial PfluDING is closely related to the eukaryotic DING protein family [2].

The binding site of PfluDING, located between the two domains, is totally buried and sequesters a phosphate molecule that copurified with the protein. The phosphate is tightly bound by 12 hydrogen bonds formed with 8 residues distributed on either side of the cleft (Fig. 3). Residues implicated in phosphate binding are T8, L9, S32, D62, R141, S145, G146 and T147. Hydrogen bonds distances range from 2.49 to 3.16 Å. The shortest hydrogen bond involves a phosphorus oxygen atom and a carboxylic oxygen from the D62 side chain. Such short bonds have also been reported in *E. coli* pstS (1IXH), *M. tuberculosis* pstS-1 (1PC3), and HPBP (2CAP) structures with distances as small as 2.43 Å. Being potentially the only hydrogen bond acceptor in the binding site cavity of PfluDING, D62 should play a key role in phosphate specificity

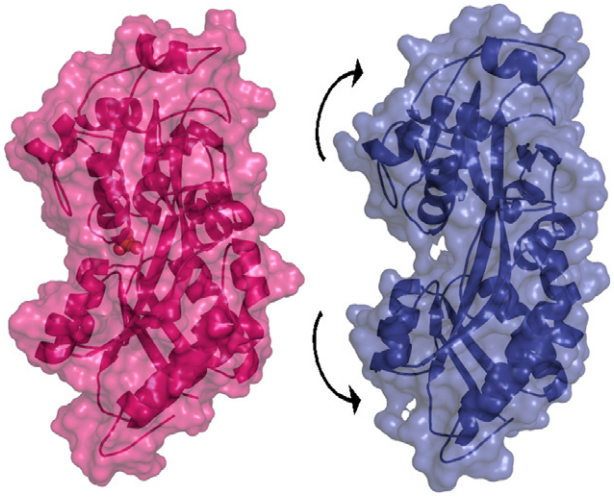

Fig. 4. The Venus "flytrap" motion. The bending motion of the Venus "flytrap" fold is presented through comparison of "closed" (wild-type) (in purple) and "open" (T141D mutant) (in blue) forms of *E. coli* PBP (pdb code 1OIB and 1IXH). Both proteins are presented in a cartoon mode with their corresponding molecular surface. The inorganic phosphate bound in the closed form is represented as balls and sticks (red).

by accepting protonated phosphate species [7]. The binding sites residues are completely identical to those identified in the HPBP structure.

Previous studies have shown that PfluDING bound ^{32}P-phosphate, corresponding to approximately one mole of phosphate per mole of protein, with a K_D of 1.7 μM [10]. The K_D values for the *E. coli* and *P. aeruginosa* pstS proteins are approximately 1 μM and 0.34 μM, respectively [7,23]. It is not immediately obvious from the structural comparisons why the PfluDING and pstS proteins should have different affinities for phosphate.

The "Venus flytrap" fold was shown to undergo large conformational changes upon ligand binding. These structures were shown to adopt a "closed" conformation whilst ligand is bound, and an "open" conformation without ligand, through a bending motion of the two domains around the hinge [22]. Yao et al. obtained a T141D mutant of *E coli* pstS (equivalent to a T147 substitution in PfluDING) with a reduced phosphate affinity [24,25]. From this mutant it was possible to obtain both "closed" and "open" forms of this protein (Fig. 4).

With the aim of obtaining similar properties, the T147N PfluDING mutant was prepared, successfully expressed and purified. The normal PfluDING protein (3.6 μg) bound 3.3×10^4 ($\pm 0.2 \times 10^4$) dpm of the radioisotopic phosphate, whereas in the same quantity of the PfluDING T147N mutant protein, phosphate binding was reduced by 85%, to 0.5×10^4 ($\pm 0.05 \times 10^4$) dpm.

Another putative mutant version of PfluDING was created, with the substitutions R141L, S145I and T147I. Although expressed in our *E. coli* system, the resulting protein was almost entirely insoluble (not shown).

Truncation of PfluDING was considered because of the discovery of the biologically-active DING protein from *H. perforatum*, which comprises 263 residues with 89% sequence identity with the N-terminal sequence of the PfluDING protein (see Fig. 1). Though the nature of the activity (inhibition of gene expression and replication in HIV-infected cells [7]) is very different from those observed with most other DING proteins, we thought it possible that such proteins might retain other activities if similarly truncated. Accordingly, we designed

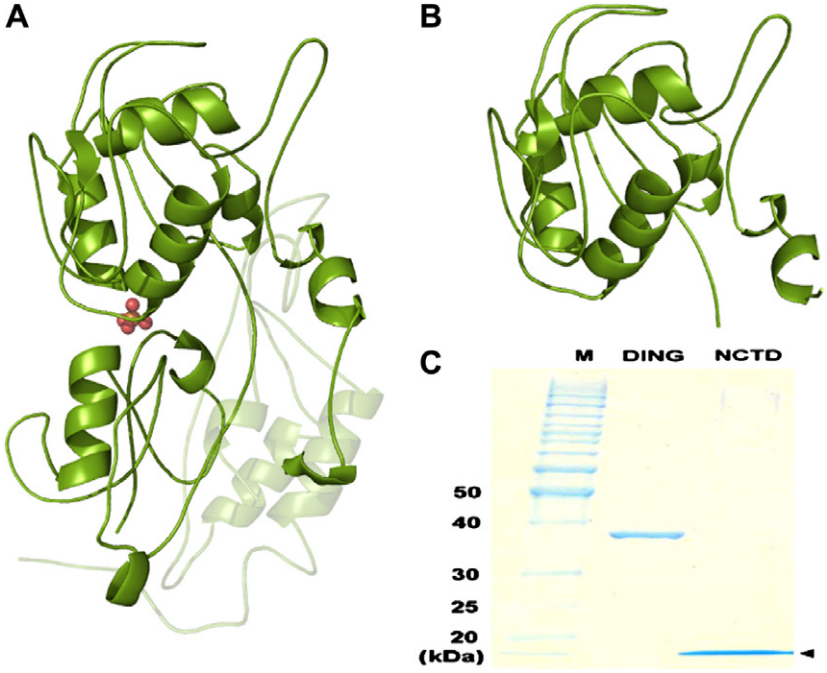

Fig. 5. N- and C-terminal truncation. (A) C-terminal truncated form (Asp1–Arg261) deduced from the complete structure of the PfluDING (truncated part is shown as transparent). (B) N- and C-terminal truncated form (Val90–Ala252) deduced from the complete structure. (C) SDS–PAGE of standard protein markers (lane M), wild-type PfluDING (lane DING) and the N- and C-terminally truncated mutant (lane NCTD).

a primer to amplify PfluDING cDNA, in conjunction with MD5, between the N-terminal codon and the codon for R261. Although the corresponding cloned, truncated protein could be readily expressed, only traces of the expected 27 kDa protein were seen, with much larger quantities of 25, 23 and 20 kDa proteins, corresponding to products of N-terminal proteolysis (not shown). It is clear from the structure (Fig. 5A) that the deletion of the C-terminal part of the "lower lobe" of the "Venus flytrap" could expose the remainder of the structure to proteolysis, either during bacterial protein processing, or during bacterial lysis and protein purification. We therefore resolved to remove the proteolytically-sensitive region by genetic truncation. Consideration of the structure of PfluDING suggested that a concomitant extension of the C-terminal truncation would effectively create a 17 kDa protein comprising just the "upper lobe" of the complete PfluDING (Fig. 5B). Thus, primers were designed to amplify the PfluDING coding sequence from the V90 codon to that for A252.

The details of the production of this NCTD protein are summarized in Fig. 5C. It has a molecular weight of 17 kDa, as expected from the genetic truncation. It retains a partial immunological cross-reaction with the native PfluDING, but cannot be detected in Western blots with an antiserum specific for the DING N-terminus (not shown). When used in phosphate-binding experiments, the extent of binding was reduced by over 92%, compared to native PfluDING (0.3×10^4 ($\pm 0.2 \times 10^4$) dpm for the truncated mutant, compared to 3.3×10^4 ($\pm 0.2 \times 10^4$) dpm for the native protein). This is unsurprising, given that four of the eight phosphate-coordinating residues (T8, L9, S32 and D62) have been eliminated, together with one side of the phosphate-binding cleft.

The mitogenic activity of the two mutants was compared with the native protein (Table 2). We have previously shown that PfluDING is mitogenic for human fibroblasts, albeit much less efficiently than is a DING protein secreted by these cells [10,12,13]. Here we show that, in the absence of bound phosphate, the protein not only retains mitogenic activity, but the activity is enhanced. This observation may indicate that the DING protein has to be in an "open" conformation for mitogenic activity, and that the "closing of the flytrap", which accompanies phosphate binding, may reduce this activity. A similar phenomenon occurs when one domain of the protein is absent, indicating that the "upper" domain, V90-A252, contains a site for mitogenic signalling. Further work is clearly needed to elucidate this effect, but the availability of the NCTD mutant should make it easier to identify the site responsible for mitogenic activity. X-ray structures of both the T147N mutant, possibly in an "open" conformation, and the NCTD mutant should also be of great interest in helping to understand the molecular mechanism of modulation of mitogenic activity.

Table 2
Relative stimulation of thymidine incorporation in human fibroblasts by native and mutated PfluDING

Reagent	Incorporation of 3H thymidine (dpm $\times 10^{-3}$)	Relative incorporation (%)
3% FCS	1.2 (±0.4)	100
PfluDING		
0.63 μM	1.0 (±0.3)	83
1.3 μM	2.3 (±0.2)	192
T147N		
0.63 μM	5.5 (±0.4)	458
1.3 μM	6.4 (±0.4)	533
Trunc.		
1.5 μM	7.3 (±0.6)	608
3.0 μM	8.2 (±0.2)	683

We previously showed that organophosphates would also bind to the phosphate-binding site in PfluDING [10]. In particular, ADP appeared to be an effective competitive inhibitor for phosphate binding. Sphingosine 1-phosphate was tested as a putative inhibitor, as this reagent has been shown to function as both an intracellular or extracellular component of signalling systems [26]. However, at concentrations of 0.1 and 1 mM, sphingosine 1-phosphate had essentially no effect on phosphate binding to the PfluDING protein. Other bioactive organophosphates, nucleotides and phospholipids are obvious candidates for future investigation in this regard.

The PfluDING structure (Figs. 2 and 3) reveals that the bound phosphate ion almost fills the buried cavity. To illustrate, the nearest crystallographic solvent molecules are situated 6 Å away from the phosphate. Accommodation of any larger phosphate metabolites would require conformational changes, presumably in accordance with the "Venus flytrap" motion. Organophosphate binding should thus induce a partial opening of the cleft, but it is not yet clear what effect this will have upon the mitogenic activity of the protein.

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