Cell. Mol. Life Sci.
DOI 10.1007/s00018-009-0006-6

Cellular and Molecular Life Sciences

REVIEW

For whom the bell tolls? DING proteins in health and disease

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Received: 1 October 2008 / Revised: 9 February 2009 / Accepted: 13 February 2009

Abstract DING proteins, identified mainly by their eponymous N-terminal sequences, are ubiquitous in living organisms. Amongst bacteria, they are common in pseudomonads, and have been characterised with respect to genetics and structure. They form part of a wider family of phosphate-binding proteins, with emerging roles in phosphate acquisition and pathogenicity. Many DING proteins have been isolated in eukaryotes, in which they have been associated with very diverse biological activities, often in the context of possible signalling roles. Disease states in which DING proteins have been implicated include rheumatoid arthritis, lithiasis, atherosclerosis, some tumours and tumour-associated cachexia, and bacterial and viral adherence. Complete genetic and structural characterisation of eukaryotic DING genes and proteins is still lacking, though the phosphate-binding site seems to be conserved. Whether as bacterial proteins related to bacterial pathogenicity, or as eukaryotic components of biochemical signalling systems, DING proteins require further study.

Keywords DING protein · Phosphate-binding protein · Phosphate deprivation · Pathogenesis · Transcriptional modulation · Bacteria · Eukaryotes

Electronic supplementary material The online version of this article (doi:10.1007/s00018-009-0006-6) contains supplementary material, which is available to authorized users.

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Published online: 17 March 2009

Introduction

DING proteins, characterised by a conserved N-terminal sequence beginning DINGGG-, and usually of 38–40 kDa, were first identified in several animal and plant tissues during the 1990s [1]. Further identifications in eukaryotes, including fungi, have continued, and a range of bacterial homologues have also been identified. In many examples, an N-terminal sequence determination is the sole basis of classification, but more extensive homology is apparent in all cases where additional peptide or DNA sequences are known. Although DING-coding sequences have been amplified from genomic or cDNA obtained from several eukaryotic tissues, no DING sequence has yet been assigned in any of the publicly accessible eukaryotic genome projects. DING proteins have been associated with a number of human disease states, but only in a few cases has the association been correlated with properties of the protein. The ability to bind phosphate seems to be one common property, confirmed by three-dimensional

structure determinations for both a eukaryotic and a prokaryotic DING protein. The significance of this ability in the eukaryotic context is unclear.

The earliest review of the field noted low-identity matches (20–30% identical) between these eukaryotic protein fragments and bacterial phosphatases and phosphate-binding proteins [1]. As systematic bacterial genome sequencing progressed in the early 2000s, it was realised that *Pseudomonas* genomes contained other sequences that were even more closely related to eukaryotic DING proteins (Suppl. Fig. 1). Although many such pseudomonad sequences represent hypothetical proteins, several have been characterised as natural or recombinant proteins. Their physicochemical and genetic properties are consistent with roles in bacterial phosphate acquisition. This situation is very different from that of eukaryotes, where many proteins have been isolated and partially characterised, but a complete protein sequence is known for only one eukaryotic DING isolate, the human phosphate-binding protein (HPBP). The corresponding genetic information is even more scarce, since the complete gene sequence is not available for any eukaryotic DING protein, which has hampered DING research. Against this background, the possibility must be considered that "eukaryotic" DING proteins arise from bacteria. It seems appropriate, then, to begin by examining the more complete picture that is presented by the prokaryotic DING and related proteins.

Bacterial DING proteins

Genes and proteins

Recent bioinformatic comparisons (Fig. 1; Suppl. Fig. 1) indicate that DING proteins are one of three families within a superfamily of bacterial phosphate-binding proteins [2, 3]. The bacterial DING proteins are 70–80% identical with eukaryotic DING proteins. The gene for one such protein, PfluDING from *P. fluorescens* SBW25, has been cloned and expressed in *Escherichia coli*. It binds a single phosphate ion, but has no detectable phosphatase activity. Its tertiary structure has been determined and closely resembles the structure of a human phosphate-binding DING protein [4, 5]. PfluDING is coded by the *psp* gene, which has a putative *pho* box, linking its expression to low extracellular phosphate levels. It is an extracellular protein, partially dependent for secretion upon the proteins coded by the *hxc* type II secretory operon [3].

The PstS proteins for the phosphate-binding component of ABC phosphate uptake systems make up the second family, and are ubiquitous in bacteria. They are coded by genes in *pho* regulons, which also code for the A, B and C proteins of the transmembrane channel, and its activating ATP hydrolase [6]. The PstS proteins are 20–25% identical in the amino acid sequence with fragments of eukaryotic DING proteins, but do not have the DINGGG- N-terminal sequence [2]. The PstS proteins are periplasmic (in gram-negative organisms) or anchored to the cell envelope. They have a characteristic "Venus flytrap" tertiary structure, with two globular domains that hinge together to form a phosphate-binding site [7]. This is broadly similar to the structures of the PfluDING and HPBP proteins.

A third family is much less well-characterised, but comprises alkaline phosphatases, which are 40–50% identical with eukaryotic DING proteins [2]. There is usually some variation from the DINGGG- N-terminal sequence. These "AP family" genes are not found in *pst* operons, but are also induced by low phosphate concentrations, and the resulting proteins are secreted into the bacterial environment. They sometimes occur in tandem in *Pseudomonas*, giving rise to two alkaline phosphatases identified as lapA and lapB (PA0688 and 0689) in *P. aeruginosa* PA01 [2, 8, 9]. There is as yet no tertiary structure for an AP protein, though the sequence similarity with PstS and DING proteins suggests a likely structural relationship.

It must be emphasised that the distinction between the first and third families of bacterial phosphate-binding proteins is largely based upon the degree of sequence identity. At this time, there is only one example of a DING protein, which is known not to have phosphatase activity, and two examples of AP proteins that are phosphatases [2–4, 8, 9]. Another common feature of DING and AP proteins, not shared by PstS proteins, is the conserved quartet of cysteine residues, forming two disulphide bonds in the known tertiary structures (Suppl. Fig. 1; refer also to subsection "Structure and function in DING proteins", below).

DING proteins, phosphate binding and bacterial pathogenesis

The affinity constant for phosphate binding by PfluDING is higher than for several bacterial PstS proteins (see below) [4]. This suggests, in conjunction with the secretory phenotype, that a putative role may be to sequester phosphate in the environment, which can then be preferentially given up to PstS at the cell surface [3]. It may be necessary for phosphate to be protein-bound throughout the uptake process, as the periplasm preferentially concentrates calcium [10], which could lead to calcium phosphate precipitation. Organophosphates can also be bound by PfluDING and could help provide cellular phosphate [4]. This phosphate scavenging role may also apply to AP family proteins, which have the additional property of hydrolysis of phosphate esters to generate free phosphate. However, it should be pointed out that, under laboratory conditions, psp^{-} mutants of *P. fluorescens* SBW25 grow normally, even in

		Origin
AP		
P. aeruginosa PA01/1	AVTGGGASLP AELYKGSADS ILPANFSY	DP; (3)
P. aeruginosa PA01/2	VTGGGASMP AKLYKGSADS ILPINFSY	DP; (3)
S. maltophila/1	VTGGGASLP ADLYKGSADS ILPANFSY	D; +
S. maltophila/2	VQGGGATMP AALYQGSPDS ILPTSFSY	D, +
Bacterial DING		
P. aeruginosa MDR13	DINGGGATLP QQLYQ	DP; (12)
P. aeruginosa MDR1*	EINGGGATLP QQLYQEPGVL TAGFAAYIGA	DP; (12)
P. aeruginosa PA14*	DINGGGATLP QQLYQEPGVL TAGFAAYIGV	DP; (12)
P. aeruginosa PA7*	TVNGGGATLP QQLYQEPGVL TAGFAAYIGV	D; (3)
P. fluorescens Pf5/1*	DINGGGATLP QPLYQTAGVL TAGFAPYIGV	D; (3)
P. fluorescens Pf5/2*	DINGGGSTLP QSYYLAPGVL TAGFAPYIGS	D; (3)
P. fluorescens SBW25*	DINGGGATLP QALYQTSGVL TAGFAQYIGV	DP; (8)
T. thermophilus	DVNGGGATLP QPLYQ	P; (17)
Human DING		
SSP	DINGGGATLP QPLYQTAAVL TAGF	P; (22)
hirudin-binding	DINGGGATLP QPLYQTSGVL TAGF	P; (25)
monkey/human CAI	DINGGGATLP QPLYQTSGVL TAGFAPYI	P; (38)
genistein-binding	DINXGGATLP QPLYQTXGVL PAGFAPYIGV	P; (35)
HPBP	DINGGGATLP QKLYLTPDVL TAGFAPYIGV	P; (31)
human PIF receptor	DINGGGATLP QKLYLIPNVL	P; (42)
human SIP	DVNGGGATLP QPLYQTA	P; (51)
prostate clone	AVTGGGASLP AELYKGSADS ILPANFSYAV	D; *
Animal DING		
B. plicatilis MIP	DVNGGGATLP QPLYQTA	P; (50)
rat, cotinine-binding	XINGGGATLP QKLYLTPNVL TAGFAPYI	P; (46)
Rat genomic fragment	VTGGGASLP PDLYKGQADS ILP	D ; *
woodchuck	DINGGGATLP QXLY	P; (47)
turkey	DINGGGATLP QHLYLTPDVL TAGFAPYI	P; (48)
L.major genome	DINGGGATLP QPLYQTSGVL TAGFAPYI	D; *
Plant DING		
SJW p27SJ	DINGGGATLP QALYQTSGVL TAGFAPYIGV	DP; (59)
tobacco	DINGGGATLP QKLYQTAGVL TARF	P; (54)
potato	DINGGGATLP QKLYQTSGVL TAGFAPYI	P; (54)
wheat	MINGGGATLP QKLYQTNGVL NGAFXP	P; (54)
sweet potato	DINGGGATLP QXLYXTP	P; (54)
Sesbania	DINGGGATLP QALYQTSGVL TAGFA	P; (57)
Jerusalem artichoke	DINXGGATLP QPLYQ	P; (56)
French bean	DVNGGGHTLP QPLYQTTVVL	P; (53)
Fungal DING		
C. albicans	DINGGGATLP	P; (65)
Colletotrichum sp.	DINGGGATLP QKLYQTSGVL T	P; (66)
G. lucidum	DINGGGATLP QKLYLTPDVL	P; (67)

Fig. 1 DING N-terminal sequences. The sequences are aligned in taxonomic groups, approximately as ordered in the text. In the "Origin" column, "D" indicates a sequence predicted from DNA data, and "P" indicates a determined protein sequence, followed in each case by the appropriate reference. *Database accession numbers. $^{+}$*Stenotrophomonas maltophilia* R551-3 AP-like genes GenBank AAVZ01000018

phosphate-limited medium, whereas *pstC*$^{-}$ mutants are disadvantaged under this condition [3].

A very recent and significant report suggests that the secretion of phosphate-binding proteins is a strong determinant of pathogenicity in *P. aeruginosa* [11]. Different strains secrete different proteins; the authors seem to use "PstS" as a generic term, but it is clear from the sequence comparisons that these include AP- and DING-like proteins of about 40 kDa, and their secretion involves the alternative type II secretion system encoded by the *hxc* operon. More surprisingly, 32-kDa proteins, resembling true, periplasmic PstS, are also secreted in some cases. Low phosphate concentrations induce this expression very strongly (5,000×) in the most virulent pathogenic strain. It is claimed that low phosphate concentrations may occur in vivo in the mammalian gastrointestinal tract, promoting the adherence and growth of the pathogens. It is shown experimentally that phosphate in the drinking water protects mice from this effect. The proteins form the major component of extended cell-surface fibres that promote bacterial adhesion to epithelial cells, as an early stage of the pathogenic mechanism. This adhesive property of DING proteins bears comparison with those of DING isolates from animal tissue (see below). Fimbriae of

Pseudomonas also confer adhesive and pathogenic properties, but 16- or 19-kDa fimbrial subunits clearly distinguish them from the structures identified by Zaborina et al. [11], as is also the case with other adhesive fibrils in gram-negative bacteria [12–14].

Microarray analysis of changes in gene expression, when *P. aeruginosa* PA01 interacts with human airway epithelial cells, indicates that PA0688 transcription is elevated over 20-fold. Other phosphate-acquisition genes are also stimulated, so this may also be a phosphate-dependent effect [15]. *P. fluorescens* SBW25 was originally isolated from cultivated sugar beet, but the use of in vivo expression technology (IVET) has shown that bacterial growth on sugar beet seedlings does not result in induction of PfluDING expression [3]. Low phosphate is at present the only known condition for bacterial DING induction.

Until recently, database searches had indicated that only *Pseudomonas* strains contain DING- and AP-like gene sequences (40% or more identity with eukaryotic DING sequences). Pseudomonads, as well as many other bacterial species, contain low-identity sequences (20–25%), which can be ascribed to the *pstS* genes of these species. DING and AP coding sequences have been successfully amplified from most, but not all (15/16), of a range of *Pseudomonas* strains by PCR, where they are approximately equally prevalent, and from *Burkholderia cepacia*, for which there is as yet no genomic precedent. Interestingly, the *P. putida* 852 strain appears to have a DING and an AP gene, reminiscent of the tandem AP genes in *P. aeruginosa* PA01 [16]. Genomic sequences from *Stenotrophomonas maltophilia* R551-3 contain two AP-like genes (GenBank AAVZ01000018; Suppl. Fig. 1). *Burkholderia* and *Stenotrophomonas* species were formerly classified as *Pseudomonas.*

Genetically uncharacterised DING proteins from bacteria

Several more diverse bacterial species have been the source of purified DING proteins, usually identified by N-terminal amino acid sequencing (Fig. 1). A recent report indicates that a DING protein with alkaline phosphatase, ATPase, nuclease and DNA topoisomerase activity has been isolated from *Thermus thermophilus* HT_8 [17]. A corresponding coding sequence has not been reported in the completed *T. thermophilus* HT_8 genome, nor in the two megaplasmids commonly found in this bacterial species [18, 19]. It seems unlikely that a mesophilic bacterial contaminant could be responsible for producing this protein under thermophilic culture conditions, and the phosphatase activity of the purified enzyme is thermostable. There is as yet no experimental evidence to link this protein to DNA repair, maintenance or replication in the host organism. A periplasmic location is inferred, by analogy with PstS proteins of gram-negative bacteria, but there is evidence that lipids enhance the phosphatase activity, which invites comparison with the membrane-anchored PstS proteins of gram-positive microbes [17].

A poly(ADP-ribose)polymerase enzyme from *Sulfolobus solfataricus* has been recently described, with a characteristic DING N-terminal sequence [20]. This is the first reported isolation of a DING protein from one of the Archaea, indicating that DING proteins have now been found in all biological kingdoms.

It can be seen that there is a developing body of evidence for a physiological role for bacterial DING proteins in bacterial phosphate acquisition and uptake, but that there are still many unanswered questions in this area, as well as indications of other possible functions. The ability of some pathogenic pseudomonads to generate DING proteins in eukaryotic organisms may account for some aspects of DING-associated diseases (see below).

Structure and function in DING proteins

Structure

The high sequence identity between DING proteins leads to the assumption that their structures are very similar, which is confirmed by the available three-dimensional models of PfluDING [5, 21] and the HPBP [22–24]. Indeed, both DING structures possess an elongated fold composed of two adjacent globular domains (Fig. 2a). Each domain is constituted by a central β-sheet core flanked by α-helices and contains a disulphide bridge (i.e. C113–C158 and C306–C359 in HPBP). Interconnected by an antiparallel two-stranded β-sheet acting as a hinge, the two domains form a deep cleft wherein is bound a completely buried phosphate molecule. This fold, known as a Venus flytrap, is very similar to those of the sixth family of solute binding proteins (SBP), which include PstS proteins [25, 26].

Structural superposition (Fig. 2b) illustrates the similarities between DING proteins, with mean square deviations (rmsd) of 0.65 Å on 366 Cα atoms between PfluDING and HPBP. They also superimpose quite well with *E. coli* PstS (rmsd of 1.88 Å on 276 Cα atoms and 1.51 Å Cα atoms on 216 with PfluDING and HPBP, respectively). In fact, the major difference between DING proteins and PstS proteins consists of the presence of four loops protruding from DING protein globular domains. The two DING structures also contain two disulphide bridges, which are absent in PstS. The four cysteine residues are conserved in DING sequences, insofar as these sequences are known (Suppl. Fig. 1).

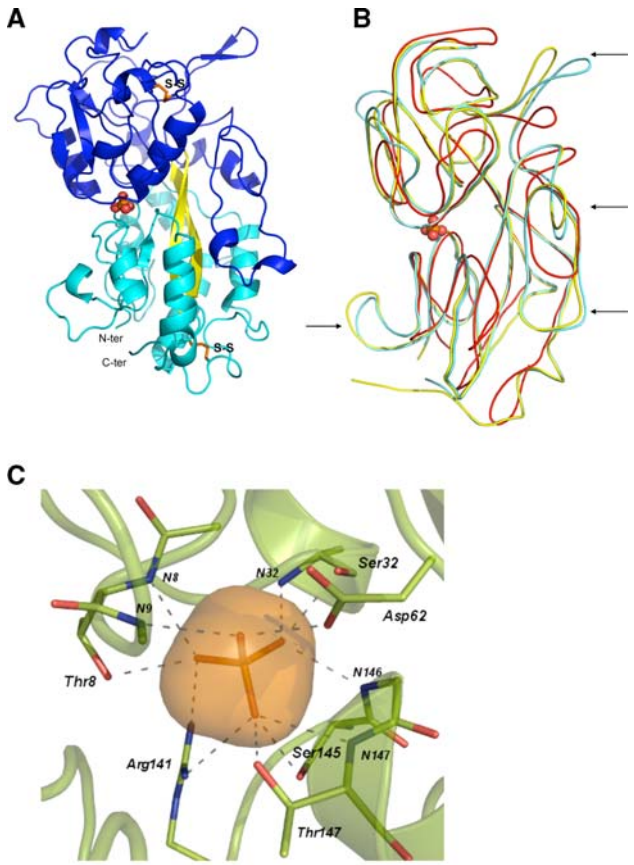

Fig. 2 Structure of DING proteins. **a** Cartoon representation of the structure of a DING protein representative: HPBP solved at 1.9 Å [23]. HPBP possess an elongated fold composed of two adjacent globular domains (in dark blue and cyan). Each domain is constituted by a central β-sheet core flanked by α-helices and contains a disulphide bridge (orange sticks). Interconnected by an antiparallel two-stranded β-sheet acting as a hinge (in yellow), the two domains form a deep cleft wherein is bound a phosphate molecule (red balls). **b** Structural comparison of different known phosphate-SBPs: HPBP is shown in cyan, PfluDING is shown in yellow, *E. coli* PstS protein is shown in red. The four protruding DING protein-specific loops are indicated by *black arrows*. **c** Phosphate-binding site of DING proteins. Residues involved in the binding of the phosphate molecule are labelled. 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. Surface is computed from the protein Van der Waals surfaces

Phosphate binding

The binding site of DING proteins is totally buried between the two globular domains and sequesters an inorganic phosphate ion that co-purifies with the protein. The binding site cavity is very small and closed (Fig. 2c). The ability of PfluDING to bind larger organophosphate molecules suggests that large conformational changes are induced. The phosphate is tightly bound by 12 hydrogen bonds formed with eight residues distributed on both sides of the cleft. The shortest hydrogen bond (2.43 Å) involves a phosphorus oxygen atom and a side chain carboxylic oxygen of an aspartic acid. Such short bonds have also been reported in *M. tuberculosis* PstS-1 (pdb code: 1PC3), HPBP (pdb code: 2V3Q) and the ultra-high resolution structure of *E. coli* PstS (pdb code: 1IXH). Surprisingly, these short bonds have been described as low energy bonds [27]. Being potentially the only hydrogen bond acceptor in the binding site cavity of these proteins, this aspartic acid should play a key role in phosphate specificity by accepting protonated phosphate species [7]. Mutation of another of the eight phosphate-coordinating residues, threonine-147, to asparagine in PfluDING reduces phosphate binding by 85% [5]. Regarding affinities for ligand, HPBP binds phosphate with submicromolar affinity [22]. Studies have also shown that PfluDING binds phosphate with a K_D of 1.7 μM [4]. The K_D values for the *E. coli* and *P. aeruginosa* PstS proteins are approximately 1 and 0.34 μM, respectively [7, 28]. It is not immediately obvious from the structural comparisons why the PfluDING and PstS proteins should have different affinities for phosphate, but this is consistent with the hypothesis, described above, that extracellular DING proteins may give up phosphate preferentially to cell-bound PstS proteins.

Although PfluDING and HPBP share 71% sequence identity, their solubilities are very different, probably determining their functions. PfluDING is expressed under conditions of phosphate limitation and secreted, and could thus be involved in extracellular scavenging of phosphates [3]. On the other hand, HPBP is a plasma hydrophobic protein, bound to the HDL-associated apolipoprotein human paraoxonase 1 (PON1) and required for its stability [29–33]. The different solubility of both proteins is nicely illustrated by their electrostatic potential patterns (Fig. 3a, b). HPBP shows large non-charged areas at the protein surface whereas PfluDING possess large patches of negative or positive charges. Because eukaryotic genes coding for DING proteins are not available, and soluble HPBP expression from a synthetic DNA in a bacterial host system remains impossible (Chabriere and Scott, unpublished results), the soluble PfluDING represents an appealing model to use for structure/function relationship studies.

In particular, the study of the "Venus flytrap" motion was attempted. Because of the difficulty of total phosphate removal, a mutation of a binding site residue was required to obtain the structure of an "open" form for *E. coli* PstS [34, 35]. This confirmed that this fold is able 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 (Fig. 3c, d) [25].

In all cases where the sequences have been determined, the eight phosphate-binding residues of DING, AP and

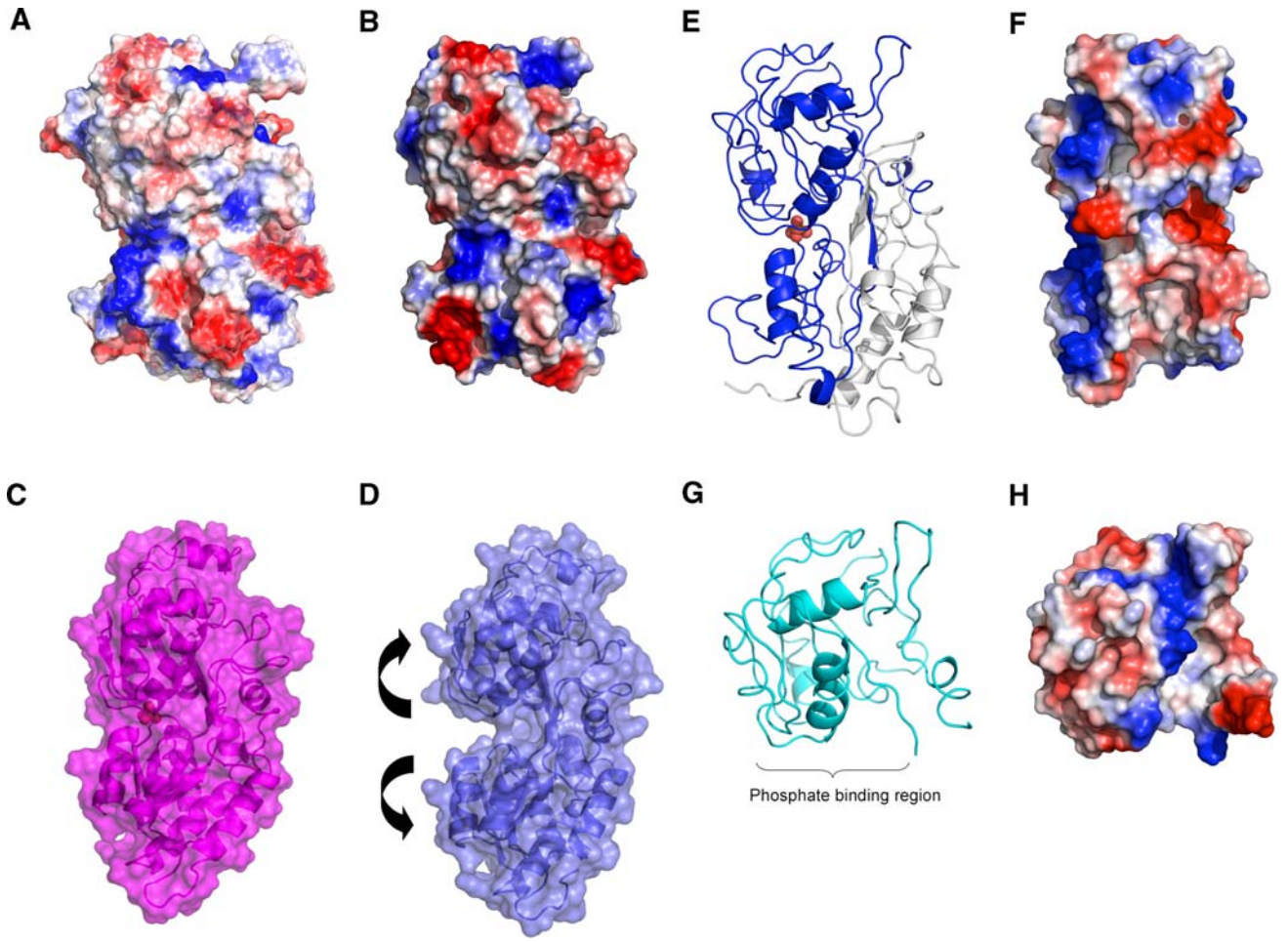

Fig. 3 Properties of DING structures. Electrostatic potential of HPBP (**a**) and PfluDING (**b**). Non-charged areas are shown in white, negatively charged areas are in red and positively charged areas are shown in blue. The large non-charged area observed in HPBP compared to the numerous charged patches at the PfluDING surface is coherent with the differences in solubility observed experimentally for both proteins. The Venus "flytrap" motion. The bending motion of the Venus "flytrap" fold is presented through comparison of "closed" (wild-type) (in purple, **c**) and "open" (T141D mutant) (in blue, **d**) forms of *E. coli* PBP (pdb codes: 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). Truncation of DING proteins. **e** $p27^{SJ}$ model (in blue) deduced from the C-terminal truncated form (Asp1–Ala252) deduced from the complete structure of the PfluDING (truncated part is shown in grey). **f** electrostatic potential of the truncated DING model. This shows that residues exposed consecutively to the truncation are mainly charged residues. **g** N- and C-terminal truncated form of PfluDING (Val90–Ala252) deduced from the complete structure. **h** electrostatic potential of the N- and C-terminal truncated form of PfluDING. This shows that residues exposed progressively to the truncation are mainly charged residues

PstS proteins are almost always identical, with very occasional conservative replacements (see Suppl. Fig. 1) [2]. This does not necessarily imply a role in phosphate uptake for all of these proteins, but it does indicate that a phosphate-triggered conformational change may be a common aspect of the biochemical function of DING proteins.

Structure–function relationship: transcriptional regulation

DING proteins have also been found in a truncated, biologically active form, as illustrated by the discovery of the DING protein from *H. perforatum* ($p27^{SJ}$), which comprises 263 residues with 89% sequence identity with the N-terminal sequence of the PfluDING protein. This protein, which inhibits gene expression and replication in HIV-1-infected cells, strongly suggests that truncated DING proteins are physiologically relevant [36]. The modelling of this truncated form based on the complete PfluDING structure is consistent with the experimental observations on the protein integrity and solubility (Fig. 3e, f). Indeed, the exposed residues are mainly polar and charged residues, which would be expected if truncated DING proteins do indeed have a cellular function. Because of this observed biological activity, truncation of PfluDING was investigated. A truncated mutant protein preserving

only the upper domain (Fig. 3g, h) was designed, expressed and purified [5]. Experiments on human fibroblast proliferation showed that PfluDING has an important stimulatory effect, that is increased for the putative open form (mutant T174N) and is maximal for the truncated form [5]. These data suggest that the "active site" for this biological activity could be between the two globular domains, as this area becomes accessible whilst the structure is open and fully accessible in the truncated form. This corresponds to the interface labelled "phosphate-binding site" in Fig. 3g.

When HIV transcription is inhibited by the $p27^{SJ}$ DING protein (see above), the initial target is the C/EBPβ transcription factor, which co-localises with $p27^{SJ}$ in the cytoplasm, preventing nuclear gene activation [36]. This phenomenon is consistent with the mitogenic effect of PfluDING and its derivatives, since sequestration of C/EBPβ will lead to enhanced proliferation in primary fibroblasts [37]. In conjunction with the truncation experiments, this suggests that the "phosphate-binding site" face of the DING upper domain (Fig. 3c) may also be responsible for C/EBPβ binding. It seems likely from these studies that transcriptional modulation could be influenced by phosphate binding.

DING proteins as binding proteins

In addition to the binding of phosphate, and of the C/EBPβ transcription factor, there is evidence for interactions between DING proteins and other ligands. In fact, ligand-affinity-based purification methods have led to the initial identification of many DING proteins. Known protein ligands for DING proteins are discussed in the following sections [38–42]. Some DING proteins also self-associate to form adhesive appendages (see above) [11]. The $p27^{SJ}$ and PfluDING experiments outlined above suggest that DING proteins are readily transported into animal cells, at least, and it seems possible that they act as co-transporters or chaperones for the uptake of other proteins.

The turkey LFPBP-40 DING protein is a lectin that recognises rhamnose [38]. Chondroitin sulphate will inhibit the interaction between the proteolysis-inhibiting factor (PIF) and its DING receptor, so is assumed to be the basis of PIF-receptor recognition [39]. The phytochemicals, genistein and cotinine, have also been identified as DING ligands [40, 41], and other small metabolic substrates must also be able to interact with DING proteins acting as enzymes (see next subsection). Binding to calcium oxalate crystals is a property of the crystal adhesion inhibitor (CAI), a renal and urinary DING protein [42], an attribute shared by recombinant PfluDING (J. Lieske and K. Scott, unpublished observations). As yet we know very little about these interactions, nor if they are influenced by phosphate binding.

DING proteins as enzymes

Most, if not all, of the reports of DING proteins with enzymic activity have demonstrated a hydrolytic activity; examples of phosphatase, phosphodiesterase and nucleotidase activity have been cited above, as being discovered in bacterial DING isolates [8, 9, 17]. Eukaryotic DING isolates have yielded cutinase and proteinase activities (see below) [43, 44]. It is tempting to speculate that some of these activities may represent different manifestations of the activity of a relatively non-specific esterase. As more DING and AP sequences are determined, it may be possible to distinguish the structural features that characterise enzymic activity.

DING proteins in health and disease

Rheumatoid arthritis and the synovial stimulatory protein

Rheumatoid arthritis (RA) is a complex multifactorial disease characterised by inflammation of the joints, synovial hyperplasia and joint erosions following the formation of the destructive pannus [45]. Rheumatoid factor (RF; autoantibodies specific to the Fc region of IgG molecules) is detected diagnostically in up to 80% of RA patients [46]. Synovial stimulatory protein (SSP) was the source of the first DING protein identified in humans. It was initially isolated from the synovial fluid of RA patients, and from the conditioned media of synovial fibroblasts from RA patients, as a 205-kDa protein (p205). Following electroelution and further SDS-PAGE, it was found that p205 consisted of three identical subunits of 70 and 60 kDa under non-reducing and reducing conditions, respectively [47]. SSP was able to activate synovial T-cells in concert with IL-2. Antibodies from RA sera cross-reacted with these proteins, whereas normal sera did not. Tryptic digestion of SSP and N-terminal sequencing revealed the characteristic DINGGG N terminus on 40-, 27- and 25-kDa fragments, and also an 11-residue peptide identical to one from to the constant region of an IgG molecule [47, 48].

The 40-kDa DING variant was also independently isolated, by binding to hirudin-agarose, from skin fibroblasts, and from normal and RA synovial fibroblasts. This protein acted as a mitogen for fibroblasts and was associated with traces of proteolytic activity. A 200-kDa SSP-like protein, cross-reacting with anti-DING antisera, was detected in normal and RA synovial fluids [44, 49]. The SSP thus appears to be a precursor of RF and also of a DING protein. However, there is insufficient evidence to suggest that SSP is the sole cause of RA, but its contribution in the pathogenesis of RA may involve sustaining inflammation of

joints as well as stimulating synovial fibroblasts to over-proliferate. Attempts to amplify cDNA or gene sequences with PCR primers based on DING peptide sequences have not identified any that are consistent with known DING sequences.

Atherosclerosis and the HPBP

Atherosclerosis is a disease of the arteries that can lead to a number of complications including myocardial infarction and stroke [50]. One of the critical processes involved in the development of atherosclerosis is the oxidation of low-density lipoproteins (LDLs) and their uptake by macrophages, which in turn leads to the formation of foam cells [51]. PON1 is considered as a protective protein against atherosclerosis [51–54]. HPBP is a DING protein that strongly interacts with PON1. In fact, HPBP was found to stabilise the biologically active conformation of PON1 [22], so it may be useful in identifying individuals with a high risk of developing atherosclerosis. One current difficulty is that although the full amino acid sequence of HPBP is known, its corresponding gene sequence is currently missing from the human genome database.

Breast cancer, genistein and DING proteins

The consumption of soy has been raised as a possible explanation for the low incidence of breast cancer in Asian countries. Reports show that genistein, a phytoestrogen from soy, can decrease the rate of tumour progression in chemically induced mouse models for mammary cancer [55, 56]. Microarray studies revealed that genistein in the diet of developing rats changed the expression of 227 genes in the uterus [57]. Affinity chromatography using genistein-coupled agarose beads isolated a 38-kDa protein with the characteristic DINGGG N-terminal sequence from the homogenate of human MCF-7 breast cancer cells [40]. Further studies will be required to determine the role of this DING protein in the aetiology of breast cancer.

Lithiasis and the CAI

Nephrolithiasis, otherwise known as kidney stone disease, involves the aggregation of predominantly calcium oxalate, and to a lesser extent calcium phosphate [58, 59]. CAI is a 39-kDa DING protein isolated from the conditioned media of monkey renal epithelial cells. CAI significantly reduced binding of calcium oxalate monohydrate (COM) crystals to epithelial monolayers, and it was also found to strongly bind to COM crystals themselves. Furthermore, Western blots revealed CAI to be present in human urine. It has been hypothesised that renal epithelial cells, in both monkeys and humans, constitutively produce CAI, coat COM crystals and prevent their subsequent attachment to epithelial cells [42].

Cachexia

Cachexia is a complex condition defined by the progressive depletion of skeletal muscle, and it causes death in approximately 20% of cancer patients [60, 61]. Proteolysis-inducing factor (PIF) is a 24-kDa sulphated proteoglycan that is able to induce skeletal muscle catabolism both in vitro and in vivo [39, 62]. PIF is a highly negatively charged molecule, and its transport into the cells seems unlikely. Therefore, it has been postulated that PIF interacts with a membrane-bound receptor to exert its effect. Recently, a DING protein was identified from membrane preparations of C_2C_{12} mouse myoblasts that showed high specificity for PIF binding (Fig. 1). Furthermore, anti-DING antibodies attenuated the effect of PIF both in vitro and in vivo [39]. A resultant hypothesis is that PIF binds to the DING receptor protein, which activates NF-κB via an intracellular signalling pathway, which in turn upregulates proinflammatory cytokines and also activates the ubiquitin-proteasome pathway for intracellular proteolysis [39, 63, 64]. There is as yet no evidence for DING protein involvement in the human PIF equivalent [65].

Neuronal signalling

Cotinine, the major derivative of nicotine, was used in affinity chromatography to isolate a 40-kDa DING protein from rat neurones. It is believed to be a receptor that carries out the non-cholinergic mechanisms that are associated with nicotine, of which cotinine is the major oxidation product [41].

Viral infection

A 36-kDa DING protein was co-purified with hepatitis B virus (HBV) particles from the serum of woodchucks [66]. Its role in the pathogenesis of the disease is not understood, but it has been suggested that this protein plays a similar role to SSP [47] and may help explain why HBV patients commonly experience arthritic pain [66].

Bacterial infection

Lipid-free polysaccharide-binding protein (LFPBP-40) was isolated from the air sac fluid of turkeys, and it plays a role in the binding of *E. coli* to epithelial cells. This hexameric DING protein binds to the polysaccharides on the surface of *E. coli* by virtue of its lectin-like properties. LFPBP-40 may aid in systemic and air sac *E. coli* infection in turkeys and chickens (Fig. 1) [38]. With the knowledge that DING

proteins are secreted from *Pseudomonas* [3], and have adhesive properties [11], the possibility that LFPBP-40 is a bacterial product deserves consideration.

Other DING proteins in humans and animals

The basis for the switch to sexual reproduction in rotifers, termed mixis, is unclear [67]. However, a recent study has identified a DING protein as a 39-kDa mixis-inducing protein (MIP) in *Brachionus plicatilis* [68]. This protein displays an identical N-terminal sequence with a human steroidogenesis-inducing protein that possesses mitogenic activity [68, 69].

There is as yet no evidence that MIP is coded by a *B. plicatilis* gene. It has been previously found that some bacteria, including various *Pseudomonas* species, are able to increase the occurrence of mixis by up to ten-fold compared to the untreated controls [70]. As outlined for the turkey DING protein discussed above, the possibility that the MIP is a bacterial product should be considered. The fact that, in humans and *B. plicatilis*, DING proteins have roles in sexual development may argue that they are endogenous proteins in each case. The alternative and very challenging, explanation is that bacterial proteins are controlling the developmental fate of eukaryotic organisms.

DING proteins in plants

In plants, the first protein with an N terminus typical of the DING family was obtained in a proteomics study of French bean cell walls [71]. As the DING family itself had not then been defined, the protein could not be related to known proteins and was not studied further.

Plant DING proteins were then fortuitously rediscovered in a study of germin-like proteins (GLPs). The gene for an *Arabidopsis thaliana* GLP, named AtGER3, was overexpressed, and the heterologous protein was purified from transgenic tobacco plants. As in *Arabidopsis*, heterologous AtGER3 assembled in oligomers that were analysed by SDS-PAGE. A 40-kDa band was shown to co-purify with the expected 23-kDa GLP and to associate very stably with it. The sequencing of its first 28 amino acids and of several internal peptides clearly indicated that it belongs to the DING family [72]. Partial purification followed by Western blotting indicated production from high-molecular-weight precursors and demonstrated the presence of proteins strongly cross-reacting with anti-DING protein antibodies in all species that were tested (potato, tomato, tobacco, wheat, sweet potato), indicating that they are ubiquitous in angiosperms [72].

GLPs are encoded by complex gene families in plants. For example, there are about 35 GLP genes in *Arabidopsis*, making it one of the largest gene families in this model organism [73]. Interestingly, GLPs share several features with DING proteins. They are very stable extracellular proteins that have been associated with many different activities and processes, but for which a precise function has yet to be defined. Some of the GLPs possess an enzyme activity, superoxide dismutase (SOD) for example, but several features point to a more general function in cell–cell communication. Unlike DING proteins, GLPs are restricted to the plant kingdom [72].

Plant DING protein genes have yet to be identified by systematic sequencing programmes. Taking advantage of the known N-terminal and internal peptide sequences, oligonucleotides were thus designed in order to obtain clones for plant DING proteins by PCR approaches. Clones were obtained from genomic DNAs of potato and the model plant *A. thaliana* (GenBank AY224598, AY741548, AY741549, AY227748) and, by RT-PCR, from tobacco mRNAs (GenBank DQ102394). None of these clones encodes a complete DING protein. Predicted proteins are highly similar to the other DING proteins and display all their typical features (Suppl. Fig. 1). The exact origin of these gene fragments remains an unsolved issue for now.

More recently, wounded Jerusalem artichoke tubers were shown to excrete various molecules, including a 28-kDa protein with a N terminus that related it to both DING proteins and *Pseudomonas* alkaline phosphatases of the phosphate-binding superfamily (see previous section). It was tightly linked to an 18-kDa SOD, and together these proteins exhibited a cytotoxic activity towards tumour cells of both plant and animal origins [74].

Sesbania grandiflora, a leguminous tree from Thailand, is valued for its edible flowers, which are also widely used for medicinal purposes. The flowers contain a DING protein, which functions as an α-glucosidase inhibitor [75].

DING protein from St John's wort and HIV replication

The whole protein extract of callus cultures of St John's wort (SJW) was found to inhibit proliferation of a human cell line. N-terminal sequencing revealed that the protein responsible for the observed activities, designated CHP-10, was a 39-kDa DING protein [76], which inhibited proliferation of human glioblastoma cells in a dose-dependent manner. Partial amino acid sequence data were used to design primers in order to clone the full-length protein. The largest clone obtained produced a C-terminally truncated 27-kDa DING protein and was named $p27^{SJ}$ [36] (Darbinian-Sarkissian, personal communication). $p27^{SJ}$ was found to dramatically reduce HIV replication in astrocytes, by interacting with the endogenous transcription factor

C/EBPβ and the essential HIV transactivator protein (Tat), which leads to their subsequent co-localisation to the cytoplasm where they are unable to influence transcription [36]. Addition of an excretion signal from the Ig-κ light chain at the N terminus, and an uptake signal from HIV-1 Tat at the C terminus, allowed the efficient transfer of $p27^{SJ}$ from donor to recipient cell lines. Successful recipient cells exhibited the same anti-HIV activity as shown previously in stably transfected cell lines [77].

The inhibition of C/EBPβ is consistent with the anti-proliferative effect seen in glioblastoma cells. It has previously been shown that C/EBPβ is upregulated in glioma patients. Moreover, knockdown of C/EBPβ using siRNA inhibited proliferation of glioma cells in vitro [78]. In HIV-1 infection, R5 variants (which utilise CCR5 as a co-receptor, formerly known as macrophage-tropic) dominate in the early stages of disease and are responsible for viral transmission [79, 80]. C/EBP proteins are required for HIV-1 replication in macrophages [79]. Therefore, $p27^{SJ}$ may be therapeutically relevant for the early stages of HIV-1 infection due to its inhibition of C/EBPβ.

Another possible mechanism of action may relate to the ability of DING proteins to act as α-glucosidase inhibitors [75], which can act as anti-HIV agents due to their ability to prevent viral fusion [81].

Current knowledge about DING proteins in plants indicates that they share the same features as other DING proteins: they possess the typical N terminus and usually exist as 40-kDa proteins. In many cases, they seem to be involved in the control of cell proliferation and cell–cell communication, whilst at least some of them also possess an enzyme activity. Most of them bind a specific ligand with high affinity. The existence of 27-kDa plant DING proteins, which retain biological activity, recalls the 27- and 25-kDa derivatives of the SSP [47], for which there is no information on physiological relevance and possible functions.

Plants thus contain proteins almost identical to proteins responsible for diseases in animals, and some of them have already been shown to have an effect on human pathogens. Because of this and because plants are more amenable than animals to genetic manipulation, they should be considered as an attractive model for studying the role of DING proteins in pathogenesis.

DING proteins in fungi

A protein with an N-terminal DING sequence was identified as a cell-surface protein of the pathogenic yeast, *Candida albicans*. The 45-kDa protein that was sequenced has been identified as part of a 165-kDa protein, with some functional similarities to integrins, which mimics, and cross-reacts with antibodies to, a human leukocyte receptor for the activated serum complement component, C3b [82] (R. Wurzner, personal communication).

Purification of cutinases (fatty acyl esterases) from two *Colletotrichum* species led to the identification of two enzymes, of which one was a 40-kDa protein and had the characteristic DING N terminus, in each case (Fig. 1) [43]. Cutinases are characteristic products of many plant-pathogenic microbes, enabling hydrolytic penetration of the waxy cutin layer that protects plant tissues.

Ganoderma lucidum is a mushroom widely used in herbal remedies in Asia. Characterisation of protein extracts has led to the identification of a 40-kDa DING protein with anti-oxidant properties (Fig. 1). These may be due, at least in part, to the presence of selenocysteine, in place of some of the cysteine residues characteristic of DING proteins [83].

DING-coding DNA sequences from eukaryotic tissues

Three DING sequences are known to have been identified in systematic genome studies, an unordered *Rattus norvegicus* genome fragment that is not identical to the rat cotinine receptor (Fig. 1; Suppl. Fig. 1), a human breast cancer-expressed sequence tag (Suppl. Fig. 1) and one from the *Leishmania major* genome project (Fig. 1; Suppl. Fig. 1). In the latter case, the sequence similarity with *Pseudomonas* sequences led to its exclusion from the assembled genome, as a likely consequence of bacterial contamination (A. Ivens, personal communication). Both it and the rat sequence have 5′ extensions (not shown in Suppl. Fig. 1), which are consistent with bacterial secretion peptides and intergenic sequences. A more recent report of a fourth cloned DNA, from a human prostate tumour, is virtually identical to the N terminus of the *P. aeruginosa* PA01 AP protein (lapA; Fig. 1; Suppl. Fig. 1).

DNA sequences coding for DING proteins have been amplified from a range of plant tissues, and from SJW callus cultures, as described above. None of these DNA clones extend beyond about 750–850 bp from the initiation codon, and none can yet be assigned to chromosomes in their tissues of origin.

Are some eukaryotic DING proteins of bacterial origin?

It has been proposed that there are no eukaryotic DING genes and that all DING isolates from eukaryotic sources represent the consequences of experimental contamination, or of symbiotic or pathogenic associations between bacteria and eukaryotic hosts. This viewpoint was first expressed by Lewis and Crowther [84], based on codon usage

analysis of several partial DING nucleotide sequences, which resembles that of pseudomonads. The arguments for and against this hypothesis have been rehearsed [2, 84] and will not be reiterated extensively here.

Although some bacterial DING proteins are known only from protein characterisation, and others are predicted from genomic sequences, there are several cases amongst the pseudomonads where genomic sequences are known to be expressed and translated as functional proteins. In contrast, there is no such certainty for any eukaryotic DING isolate so far described. Most are characterised only by partial protein sequence data. Where the DNA sequence has been obtained, either fortuitously or as a result of systematic investigation, in most cases there is no evidence for an unambiguous genomic context. No databases contain DING sequences of confirmed eukaryotic origin.

Until recently, no identical or nearly identical bacterial and eukaryotic DING isolates had been obtained, which might be expected if eukaryotic proteins were expressed from bacterial genes. However, the virtual identity (>99%), between DNA cloned from a human tumour and a bacterial AP protein gene, indicates that bacterial DNA may be present and detectable in eukaryotic tissues. The question of whether the responsible bacteria are functional, and express DING proteins, cannot yet be answered directly, though the high degree of induction of DING and related proteins, in pathogenic *Pseudomonas*, suggests that it is possible [11]. However, this being the case, we might also expect to see more AP proteins, and even PstS, as a result of bacterial secretion, but these protein types have not so far been reported in eukaryotic isolates, so far as we are aware.

On the other hand, there is evidence for the existence of high-molecular-weight DING precursor proteins, in both plants and animals, for which there is no genetic precedent in bacterial genomes [47, 49, 72]. Another notable discrepancy relates to the failure to extend DING DNA clones, derived from eukaryotic tissues, beyond 0.75–0.85 kb from the 5′ DING-coding sequence, whereas the entire 1.1–1.2 kb of bacterial DING sequences can be readily amplified and cloned [4, 11, 16]. These shorter DNA sequences correspond to the 25–27-kDa proteins that retain the characteristic DING N terminus, so are C-terminally truncated [2, 36]. It implies some unusual genetic feature that is not present in bacterial DING genes.

At this stage, it seems that eukaryotic DING genes probably exist, but this remains to be confirmed in most experimental systems. Although gaps in mammalian genomes are slowly being filled, as heterochromatin regions are sequenced [85], the most rapid prospect of further resolution in this area is for unambiguous sequence determination of one of the DING precursor proteins. In the absence of such resolution, future investigation of DING-associated disease states should consider possible bacterial involvement.

Potential therapeutic applications

Because of the propensity of the HIV virus for mutation, a triple blockade is now routinely used in therapy, and the development of new therapeutic targets may offer insurance against the future possibility of resistance to existing drugs [86]. As outlined above, there is ongoing development of recombinant $p27^{SJ}$ as a potential anti-HIV agent, and the resulting truncated DING proteins, or DING-derived peptides, may also be active against glioblastomas, and possibly other tumour types [36, 72, 76].

In the case of the CAI [42], it may have some therapeutic potential in recombinant form, and the identification of its gene, which has not yet been reported, may allow the development of diagnostic procedures. The diagnostic potential of the HPBP for atherosclerosis, by virtue of its association with PON1, has been mentioned above.

DING antagonists

Genistein may exert some of its antitumour activity by antagonising DING proteins, though the pleiotropic actions of genistein are well known [87]. On the basis of its DING-binding activity, it has been suggested that recombinant hirudin could be used as a treatment for RA for the alleviation of symptoms and to delay the onset of physical disability [88]. It has already been used in humans for its anticoagulation effect [89], and its anti-thrombin action may also reduce inflammation [90]. It seems possible that a DING antagonist might also have some potential in treating cachexia [39]. In a somewhat different example of DING antagonism, the demonstration that oral phosphate can dramatically reduce DING expression and pathogenesis in drug-resistant *P. aeruginosa* has obvious implications for human gastrointestinal infections by this bacterium [11], and directly acting, chemical DING antagonists may have similar effects.

DING proteins and their genes thus represent a worthwhile field of research, not just from the standpoint of their importance as bacterial virulence factors. Their potential as therapeutics, particularly as anti-HIV agents, is an additional inducement for their study.

Conclusions and future prospects

DING proteins have been found in all the biological kingdoms. In addition to new reports, the recent application of text-mining as a bioinformatic technique has identified

several older reports of what are now recognised as DING proteins [91].

Recent progress on the bacterial physiology of phosphate acquisition indicates that, in some circumstances, pseudomonads, in association with mammalian epithelial cells, can produce and secrete significant quantities of DING proteins. These proteins are important in heterotypic cellular adherence, and also have the potential to be taken up by the eukaryotic cells, in which they may influence signalling pathways and gene expression. In some cases, the DING proteins identified in eukaryotic tissues may have originated in bacteria as a result of such interactions, but this has not been definitively shown.

DING proteins also act as mediators in a variety of normal aspects of the maintenance, growth and development of animal cells. It is tempting to attribute these effects to endogenous DING gene expression and protein production, but we should be open to the possibility that they may sometimes represent actions of pathogenic or symbiotic bacteria.

This raises a wider question; are DING proteins uniquely bacterial products which are part of mechanisms to promote pathogenic or commensal interactions, or does their production by bacteria represent molecular mimicry of DING-based host reactions? Confirmation of the existence of DING genes in eukaryotic hosts, particularly animals, will be a necessary first step in the investigation of the latter hypothesis. It remains possible that this field will have much wider implications for our understanding of the existence and interdependence of all living organisms. Given its putative role in many different pathologies, it is important that the DING protein is studied more intensively, irrespective of its origins.

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