Minireview

Ring up the curtain on DING proteins

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Abstract DING proteins have a characteristic DINGGG- or closely related N-terminal sequence. One is found in human synovial fluid, and may be associated with rheumatoid arthritis. Other examples have receptor or signalling roles in various human and animal cells, or are involved in biomineralisation, and several of them bind to phytochemicals. As plant DING proteins have recently been discovered, we hypothesise that the DING protein-phytochemical association may represent one aspect of a ubiquitous receptor-linked signalling system. Several microbial proteins related to DING proteins have phosphatase activity, which may relate to biomineralisation in eukaryotic systems. Plant DING proteins and their microbial relatives may elicit allergic responses leading to arthritic disease.

Key words: DING protein; Rheumatoid arthritis; Cancer cell; Neurone; Phytochemical; Biomineralisation; Germin; Germin-like protein; Cell signalling; Autoimmune disease

1. Introduction

The name 'DING protein' seems to have emerged by consensus for ubiquitous but genetically elusive proteins, usually with a molecular weight of around 40 kDa, of which the N-terminal sequence is DINGGG- [1]. It was first reported as a fragment of a larger protein, found in rheumatoid arthritis (RA) synovial fluid and secreted by RA synovial fibroblasts. Closely related proteins have been reported as having receptor or signalling roles in various human and animal cells, and as components of urinary stones and gallstones. Recently, it was discovered that DING proteins are not restricted to the animal kingdom, since proteins with almost identical N-termini have been purified from several higher plant species. In addition, a variety of prokaryotic proteins all related to phosphate transport or metabolism have N-terminal sequences strongly resembling the typical eukaryotic DING protein N-terminus.

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Abbreviations: DEP, protein domain present in dishevelled, egl-10 and pleckstrin proteins; GLP, germin-like protein; HSFP, hirudin-sensitive fibroblast proteinase; PDZ, protein domain found first in postsynaptic density protein and Dlg tumour suppressor; RA, rheumatoid arthritis; SSP, synovial stimulatory protein

2. DING proteins in RA

The synovial fluid of RA patients contains a T-cell activating factor, which acts in concert with interleukin 2 [2,3]. The factor has been purified from RA synovial fluid and from the conditioned medium of cultured RA synovial fibroblasts. This synovial stimulatory protein (SSP) acts as an autoantigen, to which T-lymphocytes from RA patients respond [3–5].

In SDS–PAGE, the SSP runs as a 205 kDa protein, which appears to consist of subunits of about 70 kDa (under reducing conditions), and of about 60 kDa (under non-reducing conditions), which suggests the presence of an internal disulfide bridge. Partial tryptic digestion of the 70 kDa protein yielded fragments of 40, 27 and 25 kDa which had a common N-terminal sequence of DINGGG-. The partial digestion also resulted, in addition to these polypeptides, in an 11-amino acid peptide with 100% homology to human IgG [4]. In RA, rheumatoid factors are immunologically directed to IgM and IgG leading to a so-called seropositive RA. It is thus possible that traces of SSP-antibody immune complexes may be responsible for the observed lymphocyte activation [6–8].

Independent research on growth regulation by the leech anticoagulant, hirudin, identified a protein known initially as the hirudin-sensitive fibroblast proteinase (HSFP). It was isolated from human skin fibroblast cytoplasmic extracts, and from fibroblast-conditioned culture medium, with hirudin-agarose affinity columns. This protein had a molecular weight of about 38–40 kDa under non-reducing conditions, and an N-terminal amino acid sequence identical to the large SSP fragments [9]. Subsequent work has indicated that the proteolytic activity associated with early HSFP isolates was absent from later preparations, and may have represented a trace contaminant [1].

Internal peptide sequences predicted from the sequence of a cDNA for the 40 kDa HSFP (see below) have been used to generate two antisera, both of which cross-react with the 40 and 200 kDa proteins from fibroblasts and with the 200 kDa protein from synovial fluid. The 200 kDa protein thus seems to correspond to the SSP, and has several sequences and epitopes in common with the 40 kDa protein, now known as the DING protein [1].

DING-specific antisera have been used to show that most human synovial fluid samples, including normal and arthritis samples, do not contain the 40 kDa protein, but do possess the cross-reacting 200 kDa protein. Both the 40 and 200 kDa proteins are secreted by normal, osteoarthritis and RA syno-

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vial fibroblasts, but only the 40 kDa DING protein is found in synovial cell cytoplasm [1]. This distribution pattern is consistent with synthesis and secretion of the SSP from synovial cells, followed by proteolytic processing and re-internalisation of the resulting DING protein.

These proteins have an autocrine growth-promoting function which does not seem to involve interactions with other soluble, secreted proteins, since affinity chromatography of SSP/DING did not result in the co-purification of other proteins. It seems most likely that there is a membrane- or extracellular matrix-bound receptor for the DING protein. Internalisation into the cytoplasmic compartment after interaction with this receptor would account for the existence of the 40 kDa cytoplasmic protein.

Though it is clear that SSP/DING is not unique to RA, it may be involved in the aetiology of RA through the stimulation of T-cells. In addition, stimulation of fibroblast proliferation by SSP/DING may contribute to the synovial hyperplasia, joint swelling and the formation of the destructive pannus, which are seen in RA [10,11]. We have shown that hirudin, which binds fibroblast DING protein (see below), as well as antibodies directed against internal DING peptides, can inhibit this proliferation [1,9].

3. Other animal DING proteins

A 39 kDa protein with a DINGGG- N-terminus has been isolated as a genistein-binding protein from human breast carcinoma cell-conditioned medium (M. Belenky, H. Kim and S. Barnes, personal communication). Genistein, a soybean-derived isoflavone, is well known as an oestrogen analogue and tyrosine kinase inhibitor, but the kinetics of the growth-inhibitory effect of genistein on breast carcinoma cells indicate that these activities are not significant [12]. One suggestion is that genistein may interact with transforming growth factor β signalling pathways [13]. Genistein is also known to inhibit DNA replication and cell cycle progression, and to promote apoptosis in some cell types, so it has pleiotropic effects upon cell proliferation [14–16]. It seems possible that at least some of these effects may stem from the interaction of genistein with a secreted, autocrine stimulatory protein identical or related to the DING protein.

A 40 kDa protein, isolated from rat neurones, functions as a membrane-associated receptor for the principal nicotine metabolite, cotinine, and may be responsible for the non-cholinergic activities of nicotine [17]. The N-terminal sequence shows greater than 80% identity with the human DING sequence. This finding indicates that DING proteins have a broader scope as signalling molecules in mammalian cells. Taken together with the findings on genistein, it suggests that a number of phytochemicals may have the ability to interact with DING proteins, and thus to influence signalling to animal cells.

A 40 kDa turkey air sac fluid protein with a high degree of homology (ca 85% identity) to SSP/DING has also been reported. This protein, named LFPBP-40 (lipid-free polysaccharide-binding protein), exists as a multimer of six covalently bound subunits. It is involved in bacterial adhesion to epithelial cells in respiratory infections by its ability to bind microbial surface polysaccharides through a lectin-like activity [18].

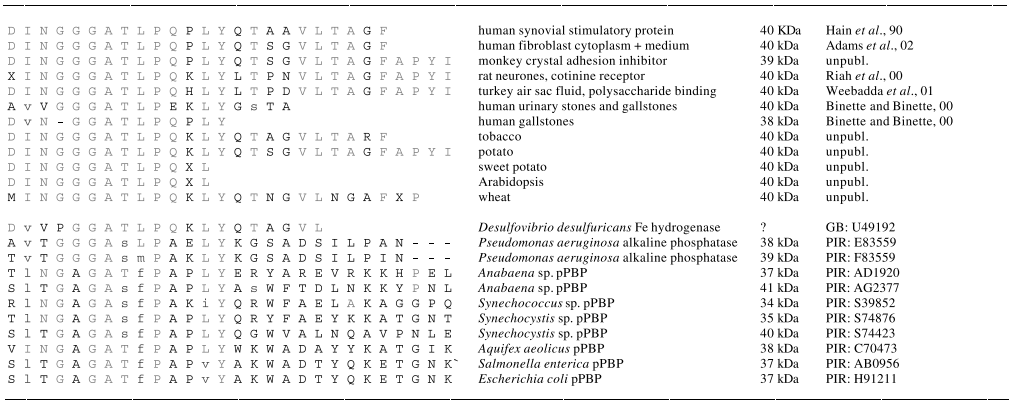
Other examples of DING proteins in animals are known, and reinforce the view that this is a family of proteins with structural similarities but divergent functions. A short N-terminal sequence of a 40 kDa protein, extracted from human urine and gallstones, has 60% identity with the synovial fluid and fibroblast DING protein sequences [19]. A 39 kDa sialoprotein called the crystal adhesion inhibitor is secreted by monkey renal epithelial cells, and was isolated by binding to calcium oxalate crystals [20]. It may act to prevent epithelial attachment of such crystals and to block kidney stone nucleation. Its sialic acid component is essential to this activity, but the polypeptide is highly homologous ($>90\%$ identity) with the SSP/DING N-terminus. Thus DING proteins act to prevent nucleation of kidney stones, but are also incorporated into such stones when they do form. These phenomena are not irreconcilable. All that can be stated with certainty at this stage is that DING proteins are implicated in the calcification of stones.

4. DING proteins in plants

Until very recently, DING proteins or DNA coding sequences had never been described in plants. However, the first plant DING protein was recently identified in a search for proteins interacting with germin-like proteins (GLPs; A. Berna and F. Bernier, unpublished observations). This yielded a tobacco extracellular 40 kDa protein, the N-terminal sequence of which has about 90% identity with the N-termini of animal DING proteins. Similar proteins have now been detected in tomato, potato, sweet potato, wheat and in *Arabidopsis thaliana*. Plant DING proteins can be found in various tissues as well as in culture medium of in vitro propagated cells (A. Berna and F. Bernier; T. Perera and K. Scott; unpublished observations).

GLPs are ubiquitous plant extracellular glycoproteins encoded by diverse gene families. Despite their name, they are not restricted to germination. In fact, GLP genes display a wide array of regulated expression patterns resulting in the presence of GLPs in all plant tissues at all developmental stages and also in response to stress conditions. The functions of several GLPs have been identified: some are enzymes (oxalate oxidase, superoxide dismutase, nucleotide-sugar pyrophosphatase/phosphodiesterase; [21]), some play a structural role in reinforcing the wall to resist pathogenic attack, whereas others act as cell-surface receptors for molecules like auxin or *Rhizobium* rhicadhesin. However, the precise function of most of these proteins remains unknown. Based on current knowledge of GLPs, it is hypothesised that they might represent a family of cell-surface proteins involved in signalling between the extra- and intra-cellular spaces [22].

The potato and *A. thaliana* 40 kDa DING proteins cross-reacted strongly with an antiserum to the fibroblast DING N-terminus and weakly with antisera against fibroblast internal epitopes. This indicates that the plant-mammalian homology is not merely in the N-terminal domains, and argues for a correspondingly high degree of functional conservation in some DING proteins. However, neither *A. thaliana* nor sweet potato DING proteins bound to hirudin-agarose columns, and there are also differences in the molecular weights of putative plant DING protein precursors, detected during isolation (T. Perera and K. Scott; unpublished observations). As in animals, there thus seem to be structurally divergent DING proteins in plants, which may also reflect functional differences.



human synovial stimulatory protein	40 KDa	Hain *et al.*, 90
human fibroblast cytoplasm + medium	40 kDa	Adams *et al.*, 02
monkey crystal adhesion inhibitor	39 kDa	unpubl.
rat neurones, cotinine receptor	40 kDa	Riah *et al.*, 00
turkey air sac fluid, polysaccharide binding	40 kDa	Weebadda *et al.*, 01
human urinary stones and gallstones	40 kDa	Binette and Binette, 00
human gallstones	38 kDa	Binette and Binette, 00
tobacco	40 kDa	unpubl.
potato	40 kDa	unpubl.
sweet potato	40 kDa	unpubl.
Arabidopsis	40 kDa	unpubl.
wheat	40 kDa	unpubl.
Desulfovibrio desulfuricans Fe hydrogenase	?	GB: U49192
Pseudomonas aeruginosa alkaline phosphatase	38 kDa	PIR: E83559
Pseudomonas aeruginosa alkaline phosphatase	39 kDa	PIR: F83559
Anabaena sp. pPBP	37 kDa	PIR: AD1920
Anabaena sp. pPBP	41 kDa	PIR: AG2377
Synechococcus sp. pPBP	34 kDa	PIR: S39852
Synechocystis sp. pPBP	35 kDa	PIR: S74876
Synechocystis sp. pPBP	40 kDa	PIR: S74423
Aquifex aeolicus pPBP	38 kDa	PIR: C70473
Salmonella enterica pPBP	37 kDa	PIR: AB0956
Escherichia coli pPBP	37 kDa	PIR: H91211

Fig. 1. Alignment of the N-terminal sequences of animal and plant DING proteins (top) and of the microbial relatives of these (bottom). Animal and plant sequences were all derived from N-terminus protein sequencing. Microbial sequences were deduced from DNA sequences and are located at or very near the predicted N-terminus of the mature protein. Protein molecular weights were either determined by gel electrophoresis (eukaryotic proteins) or predicted from the sequence (prokaryotic proteins). Amino acid residues conserved in at least 85% of the eukaryotic proteins are shown in pale grey, whereas changes considered as conservative are shown in lower case text. pPBP: periplasmic phosphate-binding protein.

5. Microbial relatives of DING proteins

Preliminary data suggest that the DINGGG sequence may be present in *Candida albicans*, demonstrating that this protein family may be highly ubiquitous in eukaryotes (R. Würzner, personal communication). It is also found in prokaryotic organisms, as a search of the databases revealed that a group of microbial proteins shares a significant homology with the first 14 amino acids of eukaryotic DING proteins. With one uncategorised exception, all these proteins are periplasmic alkaline phosphatases or periplasmic phosphate-binding proteins that display a low overall amino acid identity (Fig. 1).

6. Exogenous DING proteins and RA

Having expanded the DING protein story to include plant and microbial examples, it is worthwhile revisiting the causation of RA. Rheumatic disease has long been thought to represent an interaction of environmental agents on a background of genetic susceptibility. RA may be triggered by infections or by an allergic response. Herpesviruses (EBV; HHV-6, HHV-8), retroviruses and parvoviruses are considered possible aetiological agents in autoimmune diseases such as RA, with a particular emphasis on Sjogren's syndrome [23–25]. The link between SSP/DING and arthritis is strengthened by another recent study, which showed that the DING protein co-purified with a hepatitis virus from infected animals [26]. The significance of this finding is unclear, but it is consistent with the observation that hepatitis patients often develop arthritic lesions, and suggests that proteolytic processing of SSP to DING may occur during this process. It is possible that an immune response to a DING protein, initially presented as a bacterial antigen, or complexed with a virus, could lead to subsequent autoimmune reactions in the development of RA. In addition, DING proteins, being very stable and widely distributed in plants, could act as allergens. Interestingly, germins and GLPs are now being recognised as potential food allergens [27,28]. The immunologically functional areas in the gut, known as Peyer's patches, might be responsible for an immune response to an exogenous DING protein, which could then result in autoimmune effects involving endogenous DING protein in the synovial fluid.

The first reports of plant DING proteins were in New World plants, such as tomato, potato, sweet potato and tobacco. This initially seemed to support the controversial hypothesis that RA originated in the New World, arising in Europe as a consequence of allergic responses to newly introduced food plants [29]. Temporal and geographical variability in the epidemiology of RA is indicated by palaeopathological evidence that RA was common in the New World from 4000 B.C., but was much more rare in Europe before the 17th century and unknown in Africa before the 20th century. However, more recent work has shown that a DING protein is also present in wheat, a traditional European food crop (A. Berna and F. Bernier, unpublished observations). Antibodies to unspecified wheat proteins are common in RA patients, though elevated immune responses to other dietary proteins were also noted [30]. More detailed analysis of DING protein distribution in the plant kingdom and in various parts of edible plants will be necessary to clarify this issue.

Another possibility, consistent with the 'New World origin' hypothesis, is that inoculation by tobacco smoke in the lungs might lead to RA. Many studies support the concept that cigarette smoking increases the likelihood of the onset of RA (reviewed in [31]). When compared to individuals who have never smoked, significant increases in RA seropositivity have been reported in current smokers and in ex-smokers [32]. Like the gut, the lung is also characterised by immunologically functional sites which could respond to tobacco DING protein as an antigen. Possible interactions between cotinine and endogenous DING proteins may also be involved in the increased initiation of RA in smokers. Cotinine and nicotine are known to have effects on cell proliferation, both positive and negative, on vascular smooth muscle cells and periodontal ligament fibroblasts, respectively [33,34]. No evidence is yet available concerning synovial cells, but it seems possible that nicotine and its metabolite may also influence their proliferation.

7. DING proteins and biomineralisation

The involvement of DING proteins in the formation of gallstones and kidney stones has already been discussed, as has the fact that many of the microbial homologues act as

phosphatases. The microbial DING proteins may have functional as well as structural similarities with some of the animal proteins, since generation (by hydrolysis of organic phosphates) and immobilisation of large quantities of free phosphate is an essential part of calcification in animal systems. It is known that this process occurs in arthritic synovial fluid, where nucleoside triphosphate pyrophosphatase is thought to be the key enzyme [35,36]. It may also be significant that gallstones and kidney stones are more prevalent in RA patients [37,38]. Potential interactions of all DING proteins with calcium and phosphate ions should be systematically surveyed, to determine if this is a truly ubiquitous function.

At first sight, the association between DING proteins and calcification seems to have no direct parallel in plant biochemistry. Formation of calcium oxalate crystals in specialised vacuolar compartments is the most common form of mineralisation in plants. It is known that proteins are found in the crystals, but they have not yet been characterised [39]. It seems possible that DING proteins may be involved in this process, and this is an obvious area for further study. It should be noted that kidney stones more commonly contain calcium oxalate, as opposed to calcium phosphate [40].

8. Molecular cloning of DING coding sequences

Despite the frequency of discovery of these proteins, complete gene sequences coding for eukaryotic DING proteins have not yet been identified. This is puzzling, given the recent rapid progress with both the *Arabidopsis* and human genomes, and considering the enormous numbers of expressed sequence tags now known. Several explanations could account for the lack of a cDNA clone: very low transcript abundance, mRNA instability or rapid turnover. However, the absence of a cloned DING genomic sequence is more difficult to explain. A recent report, of a relatively low degree of overlap between the genes so far identified in the two human genome programmes, suggests that significant gaps may still exist in our knowledge of the human genome [41].

In a recent attempt at obtaining a human DING DNA clone, internal tryptic peptides from the human 40 kDa protein were used to design oligonucleotide PCR primers, which led to amplification of a putative, partial cDNA sequence of about 860 bp. About 350 nucleotides at the 5′ end of this sequence are highly homologous with the protein domain present in dishevelled, egl-10 and pleckstrin proteins (the DEP domain of the dishevelled protein); the remainder, apparently a continuation of the same open reading frame, codes for a novel sequence [1].

Dishevelled is a ubiquitous gene family involved in development and growth regulation. Its protein product acts as an intermediate and as a branch point in intracellular signalling pathways between a G-protein-coupled cell-surface receptor, a member of the frizzled family, and the glycogen synthase kinase 3b or the JNK kinases [42]. The multiplicity of protein interactions suggests that the dishevelled protein may act as a 'scaffold protein' in bringing together several elements of signalling pathways. In addition to its DEP domain, it also has multiple protein domains found first in postsynaptic density protein and Dlg tumour suppressor (PDZ domains) and a pleckstrin domain. The DEP domain of the dishevelled protein is found in a variety of other proteins. It is a globular domain, comprising about 80 amino acids, which interacts with protein kinases, and may have a role in GTP–GDP exchange reactions [43]. It is not known if the SSP/DING protein has PDZ or pleckstrin domains, but the DEP domain may imply a possible role in signalling reactions through interaction with a protein kinase.

9. Hypothesis: a ubiquitous signalling role for DING proteins

In animals, DING proteins seem to be associated with important signalling functions, possibly through interactions with specific ligands in the extracellular space. Up to now, genistein and cotinine as well as bacterial surface polysaccharides have been identified as DING ligands. Much less is known at present about the possible roles of plant DING proteins, but functional conservation would suggest that they may also be involved in autocrine or intercellular signal transmission.

Although little work has as yet been done on ligand binding by plant DING proteins, it may be significant that genistein and cotinine are plant products or derivatives thereof. Plant DING proteins could themselves function as binding proteins or transporters of endogenous or extraneous bioactive metabolites, analogous to the known auxin-binding proteins [44]. Interaction of these metabolites with the homologous human or animal DING proteins could explain their diverse pharmacological actions. An analogously interacting system has been proposed by McLachlan and coworkers [45], who have shown that flavonoids, involved in signalling between legumes and their rhizobial symbionts, also act as 'endocrine disrupters'. Genistein and the related isoflavones, apigenin and chrysin, are active in this system. Interestingly, some of the plant GLPs, with which DING proteins seem to associate in vivo, have been identified as auxin-binding proteins while others bind a *Rhizobium* protein, named rhicadhesin, that is involved in the first step of the bacterial root recognition process [46].

Logical extrapolation of this hypothesis suggests that the genistein–DING interaction, which inhibits breast carcinoma proliferation, antagonises a similar interaction between DING and a human cell-derived metabolite with growth-promoting properties. Similarly, in neurones, the DING protein would interact with a similar metabolite to modulate neurotransmitter functions, and this interaction would be inhibited or displaced by a cotinine–DING interaction [17]. A simpler variant of the hypothesis would be that the mammalian DING proteins no longer bind an endogenous metabolite as part of their signalling function, but retain an ancestral binding site that is targeted by these plant metabolites.

Some of these signalling pathways are linked to the activation of cell growth, and appear to contribute to the inappropriate proliferation, which is a feature of both cancer and RA [47]. A better understanding of these pathways may also lead to the discovery or development of DING-binding ligands with potentially greater therapeutic value.

Much work will be needed to fully understand the diverse functions of DING proteins, but at this point it is tempting to speculate that they represent important partners in novel signalling pathways common to plants and animals, as well as play a role in biomineralisation processes.

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