Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1744 (2005) 234–244

BIOCHIMICA ET BIOPHYSICA ACTA BBA

http://www.elsevier.com/locate/bba

Functional properties of a recombinant bacterial DING protein: Comparison with a homologous human protein

Ken Scott*, Liyun Wu

School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

Received 14 November 2004; received in revised form 28 January 2005; accepted 15 February 2005
Available online 7 March 2005

Abstract

DING proteins are highly-conserved proteins with poorly-defined cell-signalling roles in mammals. Conserved homologues are also commonplace in plants, though not as yet functionally characterized. Poor availability of the proteins, and a lack of genetic structure, hamper progress in elucidating the roles of these eukaryotic DING proteins, but highly-homologous hypothetical DING proteins have recently been identified in *Pseudomonas* genomes. We have cloned and expressed a DING protein from *P. fluorescens* SWB25 in *Escherichia coli*. The recombinant protein, and its natural human homologue, act as phosphate-binding proteins, as predicted by structural homologies with other bacterial proteins. The recombinant protein also displays other functional similarities with mammalian DING proteins, in that, like the human version, it acts as a mitogen for cultured human cells, and can bind cotinine, known to be a binding ligand for a rat neuronal DING protein.

Keywords: DING protein; Cell-signaling; *Pseudomonas fluorescens*; Human; Mitogen; Phosphate-binding

1. Introduction

DING proteins were named for a characteristic N-terminal amino acid sequence (DINGGGATL...), highly conserved in animal and plant isolates, but more variable in a range of microbial proteins [1]. In the majority of eukaryotic examples, the N-terminal regions, of up to 25–30 amino acid residues, are 80–90% identical in sequence. Most of these proteins have molecular weights of about 40 kDa, but, as yet, no complete gene or protein sequences have been available for the eukaryotic isolates. It is thus possible that they have in common only a conserved N-terminal "DING domain", rather than conserved elements of a complete sequence.

Human DING proteins were first identified on the basis of lymphocyte stimulatory activity in synovial fluid [2], and their presence in gall- and kidneystones [3]. Further evidence for roles in stimulation of cellular proliferation [4–7], in neuronal signalling [8], in biomineralization [9], in promoting bacterial adhesion to epithelial cells [10] and as a possible viral receptor [11] has been obtained, in experiments with a range of human and animal cells and tissues. Plant DING proteins associate with germin-like proteins, but as yet no functional role has emerged [1]. Although eukaryotic DING proteins have been detected in cytoplasmic extracts, most of these examples have been identified in extracellular or cell-surface localizations.

Most bacterial homologues of the DING proteins exhibit rather lower degrees of sequence similarity, and often also have some structural homology with phosphate-binding proteins or phosphatases [1]. Microbial proteins with these properties may be periplasmic proteins, at least in Gram-negative bacteria, where periplasmic binding proteins act to concentrate ions and other metabolites from the environment, prior to uptake by cytoplasmic membrane transporters. These proteins form the largest functional class in the periplasm, followed by catabolic enzymes, including phosphatases and other hydrolases, which may act to

* Corresponding author. Fax: +649 3737668.
E-mail address: k.scott@auckland.ac.nz (K. Scott).

0167-4889/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2005.02.003

degrade bacterial and environmental products into re-usable monomers, again for uptake by cytoplasmic membrane transporters [12].

When hypothetical protein sequences with high homology to the DING protein (ca. 70–75% identity) were identified in *Pseudomonas aeruginosa* PA14 and *P. fluorescens* SBW 25 (F. Bernier and X. Zhang, personal communications), it seemed significant that they also displayed strong sequence similarity to phosphate-binding proteins, and contained putative periplasmic targeting signal peptide sequences.

We here report the cloning, expression and characterization of the DING protein from *P. fluorescens* SBW 25, with particular emphasis upon functional properties that could relate to those established for DING proteins from other species. Phosphate binding is a likely putative function, and, if general in DING proteins, may be linked to biomineralization [1]. In two cell-signalling situations, the DING proteins interact with the aromatic phytochemicals, genistein and cotinine, respectively [7, 8], and it has also been suggested that a common role in phytochemical ligand binding could account for the conservation of structure between plant and mammalian DING proteins [1].

A preliminary account of this work has been presented as a poster at Bioscience 2004, a Biochemical Society (UK) conference (abstracted in Biochemical Society Transactions [http://www.biochemsoctrans.org/bst/meet.htm]; abstract no. E312).

2. Materials and methods

2.1. Cell culture and mitogenic assays

Cultures of *P. fluorescens* SBW 25 and *Escherichia coli* BL21 (DE3) were gifts from Professor Paul Rainey and Dr Shaun Lott, respectively. They were maintained on LB broth agar plates and grown in LB broth liquid cultures. Human skin fibroblast cells (strain WI-38) and human cervical carcinoma cells (strain HeLa) were maintained and grown as previously described [6]. For mitogenic experiments, stimulation of tritiated thymidine uptake was measured, as previously described [13]. Human DING protein was prepared from fibroblast-conditioned medium or fibroblast cell lysates as previously described [6].

2.2. Cloning and expression of bacterial DING protein

The primers for exploratory PCR reactions were based on nucleotide sequences from the putative DING gene in *P. fluorescens* SBW 25, obtained from the PseudoDB database (http://pseudo.bham.ac.uk). The forward primer (PFD1) was 5′-GAC ATC AAT GGC GGT GGT GCC-3′, and 5′-CAG CGG ACG ACC GAT ACC GTT-3′, was used as the reverse primer (PFD2). To facilitate cloning in the pET-22b(+) expression vector, the forward PCR primer (MD5) for reamplification was 5′-CAA CCA TGG AC ATC AAT GGC GGT GGT GCC-3′ (incorporating an Nco I restriction site), and the reverse primer (MD6) was 5′-CGT CTC GAG CAG CGG ACG ACC GAT ACC GTT-3′(with an Xho I site). Colony PCR was used for bacterial DNA amplification, using *Taq* PCR Master Mix (Qiagen), with reaction conditions as follows: initial DNA denaturation at 94 °C for 3 min, followed by 30 cycles (94 °C denaturation for 45 s, 65 °C annealing for 45 s and 72 °C polymerase extension for 1 min), with a final 72 °C extension for 5 min. PCR products were analysed by 1% agarose gel electrophoresis [14].

DNA bands, excised and purified from agarose gels, were sequenced by the DNA Sequencing Unit of the School of Biological Sciences, using the PCR primers as sequencing primers.

The PCR product (500 ng) or pET-22b(+) vector (Novagen; 3 μg) were cleaved with Nco I and Xho I, ligated and cloned into *E. coli* DH5α. Positive clones were identified by colony PCR, and the insert cDNA sequence checked, using the T7 promoter and T7 terminator primers for forward and reverse reactions, respectively [14]. The host strain for protein expression was *E. coli* BL21 (DE3), rendered competent by electroporation. Plasmid-transformed colonies were again checked by colony PCR, and then for DING protein expression by small-scale LB broth culture of positive colonies. Bacteria harvested before and after induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside were tested by SDS-PAGE for the expression of recombinant DING protein under different conditions. Induction was at 37 °C for 3 h, at 28 °C for 3 h or overnight, and at 15 °C overnight.

SDS-PAGE was carried out in precast 12% gels (Biorad Ready Gel). Protocols for Western blotting were as previously described, using a rabbit antiserum to a conjugated human DING protein N-terminal peptide [6].

2.3. Affinity chromatography

Transformed and induced *E. coli* BL21 (DE3) cells collected from a 500 mL culture were sonicated, and the cleared supernatant was loaded onto a column packed with 2 mL of Ni-NTA beads (Qiagen), at a flow rate of 0.5 mL/min [15]. The column was washed with 30 mL of 20 mM Tris–HCl buffer (pH 8.5), containing100 mM KCl, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol and 20 mM imidazole, then with 10 mL of 20 mM Tris–HCl buffer (pH 8.5), 1 M KCl, 5 mM 2-mercaptoethanol and 10% (v/v) glycerol and then 10 mL of the starting buffer. The bound protein was eluted with 20 mM Tris–HCl buffer (pH 8.5), containing 100 mM KCl, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol and 100 mM imidazole. Fractions of 1 mL were collected and A_{280} was determined for each fraction. Fractions of interest were then combined and dialysed against de-ionized water for 24 h with at least three changes of water at 4 °C. The dialysed sample was freeze-dried and