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Conformational stability studies of the pleckstrin DEP domain: definition of the domain boundaries

Abdelhakim Kharrat [a], Stefania Millevoi [a], Elena Baraldi [a], Chris P. Ponting [b], Peer Bork [a,c], Annalisa Pastore [a,*]

[a] *EMBL, Meyerhofstr. 1, D-69012 Heidelberg, Germany*
[b] *The Old Observatory, Oxford Centre for Molecular Sciences, University of Oxford, Fibrinolysis Research Unit, South Parks Road, Oxford OX1 3RH, UK*
[c] *Max-Delbruck-Center for Molecular Medicine, Berlin-Buch, Germany*

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Abstract

Pleckstrin is the major substrate of protein kinase C in platelets. It contains at its N- and C-termini two pleckstrin homology (PH) domains which have been proposed to mediate protein–protein and protein–lipid interactions. A new module, called DEP, has recently been identified by sequence analysis in the central region of pleckstrin. In order to study this module, several recombinant polypeptides corresponding to the DEP module and N- and C-termini extended forms have been expressed. Using circular dichroism (CD) and nuclear magnetic resonance (NMR) techniques, the domain boundaries have been determined that yield a soluble and folded pleckstrin DEP domain. This comprises 93 amino acids with an α/β fold in agreement with secondary structure predictions. Stability studies indicate that the regions surrounding the DEP domain do not contribute to its stability suggesting that the phosphorylation sites at S113, T114 and S117 are in an unstructured region. Identification of the regions of pleckstrin that are folded shall facilitate determination of its structure and function.

Keywords: Pleckstrin; Domain boundary; Modular protein; Thermal stability

1. Introduction

Agonist-induced platelet activation is closely associated with phosphorylation of several proteins following activation of the protein kinase C (PKC) [1]. Among the major substrates of PKCs is pleckstrin, a 40 kDa protein solely found in cells of hematopoietic origin [2,3]. Although phosphorylation of pleckstrin has long been known to be closely associated with the secretion of platelet granule contents in response to physiological agonists [1,4,5], the functions of this protein in platelets are still not well understood.

Sequence analysis shows that pleckstrin is a mosaic protein containing copies of two different types of protein modules: the multidomain character of pleckstrin is typical of signalling proteins. Two internal repeats called PH (for Pleckstrin Homology) reside at the N- and C-termini of pleckstrin, respectively [2,6]. PH motifs have been found in over 100 proteins, including a number of signaling and struc-

* Corresponding author. Present address: National Institute for Medical Research, The Ridgeway, London NW7 1AA, UK. Fax: +44 181-9064477; E-mail: apastor@nimr.mrc.ac.uk

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tural proteins [7–13]. These proteins include phospholipase C enzymes, guanine nucleotide exchange proteins, β-adrenergic receptor kinases, β-spectrin and dynamin. The structures of PH domains from several of these proteins have now been solved. Functional studies have also shown that the PH domain family covers a variety of functions including the ability to bind proteins and phospho-inositol derivatives (for review see Ref. [14]).

Recently, identification of a second domain type in the region intervening between the two PH domains relaunched discussion of the role of this protein in the control of G-protein signaling pathways [15]. The new module was called DEP from the initials of the three proteins, dishevelled (Dsh), egl-10 and pleckstrin, in which the module was initially identified. While accumulating data is helping to understand the functional role of the PH module, little is known about the structure and function(s) of DEP. The observation that DEP is not always found in association with the PH domain strongly supports the hypothesis that the two modules have independent functions. In pleckstrin, the presence of three protein kinase C phosphorylation sites in the intervening sequence between the first PH domain and the DEP module [16] suggests that Ser/Thr phosphorylation of pleckstrin might act as a switch that regulates an alternative or concerted activity of the three domains.

In order to characterize the DEP module and its role in pleckstrin, we have undertaken a structural and functional study of recombinant fragments that encompass the pleckstrin DEP domain. This approach relies on the possibility of obtaining modules as autonomously folded units. However, correct identification of domain boundaries is as essential as not always straightforward. Since intron–exon junctions are not generally conserved in intracellular domains, other criteria are required for domain boundary determination such as limited proteolytic digestion or multiple sequence alignment methods.

We report here a structural analysis of a DEP construct corresponding to boundaries previously predicted from sequence analysis [15] and additional constructs with extensions at their N- and C-termini. By a combined use of protein engineering, nuclear magnetic resonance (NMR) analysis and thermal denaturation monitored by circular dichroism (CD) spectroscopy, we identify the boundaries necessary to obtain a pleckstrin DEP module that is folded and thermostable.

2. Results

2.1. The originally proposed pleckstrin DEP sequence is insufficient to produce a folded polypeptide

A recombinant DEP polypeptide, called DEP3.4, was initially produced by expression in *Escherichia coli* (Table 1). The construct boundaries were designed on the basis of a sequence analysis of the whole DEP family [15]. However, although the polypeptide was expressed in the soluble fraction and was readily purified, it produced a far-UV CD spectrum typical of random-coil peptides with a minimum

Table 1
Summary of the behaviour of the DEP fragments characterized in this study

Constructs	Boundaries	T_m (°C)	Behaviour
DEP3.4	M132–D221	[a]	aggregation, degradation
DEP2.4	P121–D221	58	aggregation, degradation[b]
DEP1.4	G106–D221	61	aggregation, degradation
DEP1.5	G106–N229	64	aggregation
DEP3.5	M132–N229	66	stable
DEP2.5	P121–N229	62	aggregation[c]
DEP2.6	P121–G243	62	slow degradation

[a]The construct undergo a non-cooperative transition.
[b]This construct was also expressed with much lower yields as compared to the others.
[c]Appearance of NMR resonance splitting was observed after ≈ 1 week.
In the first and second columns are indicated the names of the constructs according to an arbitrary convention (see Fig. 3) and their sequence boundaries as referred to the human pleckstrin full-length sequence (P47_human). The melting point (T_m) and the behaviour of the construct are reported in columns 3 and 4, respectively.

at approximately 200 nm (data not shown). A thermal denaturation curve that followed the ellipticity at 215 nm as a function of the temperature showed that the peptide does not undergo a cooperative unfolding transition confirming that DEP3.4 is unfolded. This result is in agreement with one-dimensional (1D) NMR data. The spectrum of DEP3.4 is typical of a protein that is partially aggregated and unfolded: resonances are not disperse and line-widths are relatively broad (Fig. 1a). The domain boundaries suggested by multiple sequence alignment therefore are not sufficient to lead to a folded domain. Three possibilities were considered: (i) the pleckstrin DEP domain includes contiguous regions that were not identified in the initial sequence similarity search and that may or may not be conserved among the whole DEP family; (ii) the DEP domain does not form a stable globular domain; (iii) the region requires distal regions of the molecule, such as the PH domains, in order to adopt a folded conformation. The latter two hypotheses are rather unlikely since the pattern of conservation among DEP sequences strongly suggests a structured domain with multiple secondary structural elements and a hydrophobic core. Furthermore, DEP sequences are also present in proteins that lack the PH domain and have very diversified architectures [15]. The first hypothesis was tested by producing constructs with systematic amino- and/or carboxy-terminal extensions relative to the original DEP consensus (Table 1). All constructs resulted in expressed and soluble polypeptides. The polypeptides migrated on SDS-PAGE gels as expected from the predicted molecular mass. These findings were confirmed by mass spectrometry. Far-UV CD spectroscopy yielded spectra typical of a folded protein with minima at approximately 208 and 222 nm (Fig. 2). However, the constructs displayed different properties with respect to their tendencies to aggregate and/or degrade (Table 1). Two of the samples (DEP1.4 and DEP1.5) partially precipitated soon after purification. Others started precipitating and/or degrading after a few days. Degradation was observed on the SDS-PAGE as a smear that increased with time. The only samples which proved to be stable during a period of time of several weeks without significant aggregation and/or degradation were DEP2.5 and DEP2.6. Lack of aggregation for these two constructs was also confirmed by native

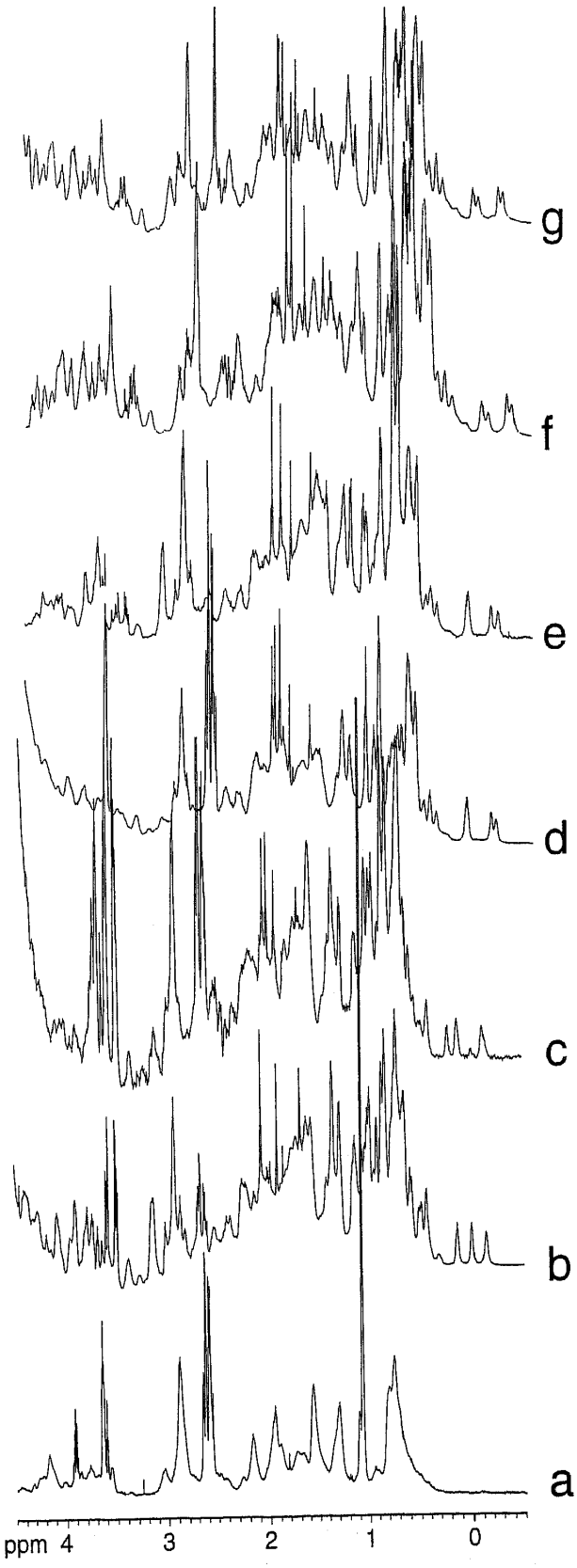

Fig. 1. One-dimensional NMR spectra of the seven recombinant constructs discussed in the paper. From bottom to the top, the spectra correspond to the following constructs: (a) DEP3.4, (b) DEP1.4, (c) DEP2.4, (d) DEP2.5, (e) DEP1.5, (f) DEP2.6, and (g) DEP3.5. The sharp peaks at 3.6 and at 2.6 ppm belong to the buffer or to other small molecular weight impurities.

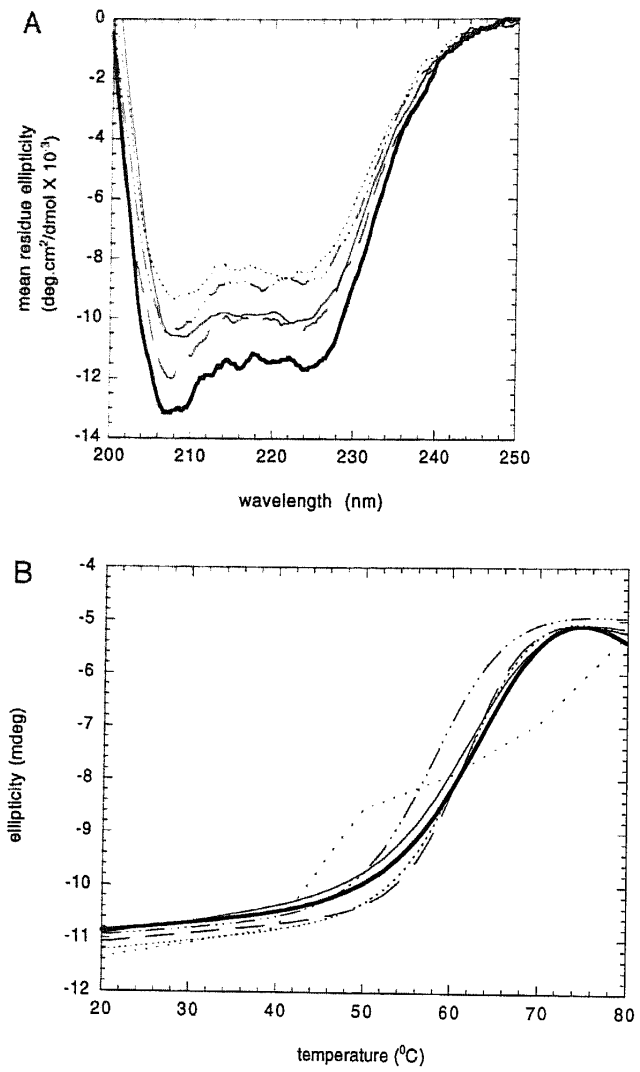

Fig. 2. a) Far-UV CD spectrum of the six folded constructs: DEP3.5 (shaded bar), DEP2.6 (— — —), DEP2.5 (————), DEP2.4 (—·—·—), DEP1.4 (· · ·). (b) Thermal denaturation curves of DEP constructs monitored by far-UV CD. For sake of clarity, only the curves of best-fit obtained from the non linear regression analysis of the normalized data are shown (see Ref. [17]). In addition to the constructs above, the non-cooperative curve of DEP3.4 (---) was added. This was recorded as single points and interpolated.

gels on which they migrated as monomeric species also after several weeks.

2.2. *One-dimensional NMR spectra indicate the minimal length necessary to produce a stable hydrophobic core*

One-dimensional NMR spectroscopy was used as a tool to analyse the structures of the different constructs and their properties (Fig. 1). The spectra show features typical of folded proteins: an appreciable dispersion of the resonances over several part per million (ppm), resonances around 5 ppm and 0 ppm, respectively, typical of α-protons involved in β-sheet conformations and of ring current shifted aliphatic protons close in space to aromatic groups. Ring current shift effects directly reflect formation of the hydrophobic core as they result from persistence of a spatial relationship between aliphatic and aromatic protons. Preservation of the same ring current shift pattern among different constructs therefore was taken as a measure of preservation of the hydrophobic core. The six samples could be divided into two sets: all those containing the region between S222 to N229 of the full-length protein have essentially the same pattern of resonances between 0.6 and -0.20 ppm (constructs DEP1.5, 2.5, 3.5 and 2.6). The only minor difference is that at 27°C the two peaks at 0.15 ppm collapse to a single resonance in constructs 1.5 and 2.5, suggesting a minor structural difference. The presence of two peaks at the same resonance in these two constructs could be confirmed by decreasing the temperature of the samples by 5°C. C-terminal addition of 14 residues (DEP2.6) does not alter the pattern but produces the appearance of sharp peaks at ≈ 0.5 ppm which could arise from the presence of an unfolded floppy tail in co-presence of the folded domain.

The second set comprises constructs which terminate at D221 and N-terminally extended (constructs 1.4, and 2.4) have different ring current shift pattern (for instance the peaks at 0.6 ppm in Fig. 1) suggesting that the extent of the hydrophobic core is reduced. As with DEP2.6, N-terminal extension does not alter the ring current shift pattern but results in the appearance of sharp signals. Apart from 3.5 and 2.6, all the other constructs demonstrated a tendency to nonspecific aggregation; precipitation and/or degradation was observed over the time by detection of the appearance of new species, e.g., the appearance of a second peak for the indolic group of the only tryptophan in the sequence in the 2D spectra of 2.5 (data not shown). This behaviour may be explained by the presence of exposed hydrophobic groups in these constructs which become buried only upon C-terminal extension. Contacts involving F225 or F226 are most likely responsible for the additional ring current shifts.

We conclude that the sequence that represents a folded DEP domain module in pleckstrin encompasses amino acids from M132 to N229.

2.3. The regions surrounding the DEP module are unfolded and have only minor influence on the stability of the domain

In order to clarify the role of the regions surrounding the DEP domain, the stability of the different constructs was investigated. Thermal unfolding studies as followed by far-UV CD spectroscopy show a single folded-to-unfolded transition for all the N- and C-terminally extended domains. Results of studies investigating the thermal denaturation properties of the constructs are summarized in Table 1. The different polypeptides enclosing the DEP module show only minor variations in their thermal stability. The melting temperature of DEP3.5 is the highest of the constructs even when considering experimental errors (Table 1). These data suggest that the regions surrounding the DEP domain are unfolded and, under our experimental conditions, have a slightly destabilizing effect on the DEP domain. Such an effect might be reduced or disappear in the context of the whole protein.

2.4. A sequence analysis of the DEP family shows no conservation of the C-terminal extension

To assess whether these findings might be expected in all DEP family domains, previously and

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                                                                    58
P47_HUMAN       1 MEPKRIREGYLVKKGSVFNTWKPMWVVLLEDGIEFYKKKSDNSPKGMIPLKGSTLTSP
P47/CHICK         .....IREGYLVKKGSMFNTWKPMWVVLLEDGIEFYKRKSDNSPKGMIPLKGSTINSP
                  ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++

                                                          1->       118
P47_HUMAN       CQDFGKRMFVFKITTTKQQDHFFQAAFLEERDAWVRDINKAIKCIEGGQKFARKSTRRSI
P47/CHICK       CQDFGKRMFVFKLTAAKQQDHFFQASYLEERDAWVRDIKKAIQCIDGGQRFARKSTRKSI
P47L/HUMAN      ..........STLKTRTSTEYFLEACSREERDAWAFEITGAIHAGQPGKVQQLHSLRNSF
P47L/PIG        ....................YFLEACSREERDAWAFEITGAIXAGQPGXVQQLHILKNSF
                ++++++++++++++++++++++++++++++++++++++++++++++++        ** *

                  2->          3->                                      178
P47_HUMAN       RLPETIDLGALYLSMKDTEKGIKELNLEKDKKIFNHCFTGNCVIDWLVSNQSVRNRQEGL
P47/CHICK       RLPETINLSALYLSMKDPEKGIKELKLEKDKKVFNHCFTGTAVIDWLVSSNSIRNRKEGX
P47/MOUSE       ............................KVFNHCLTGSGVIDWLVSNKLVRNRQEGL
P47L/HUMAN      KLPPHISLHRIVDKMHDSNTGIRSSPNMEQGSTYKKTFLGSSLVDWLISNSFTASRLEAV
P47L/PIG        KLPPHISLHRIIDKMHDSSSGIRPSPNMEQGSTYKKTFIGSSLVDWLISNSFAASRLEAV
P47L/MOUSE      ........SNIVDKMHDTSTGIRPSPNMEQGSTYKKTFLGSSLVDWLISSNFAASRLEAV
                        ----------------------------------------------------

                                                        <-4     <-5     237
P47_HUMAN       MIASSLLNEGYLQPAGDMSKSAVDGTAENPFLDNPD-AFYYFPDSGFFCEENSSDDDVIL
P47/CHICK       MLASSLLNEGYLQPA.....................................................
P47/MOUSE       MISASLLSEGYLQPAGDLSKNAADGIAENPFLDSPD-AFYYFPDSGFFCEENSSDDDVIL
P47L/HUMAN      TLASMLMEENFLRPVGVRSMGAIRSGDLAEQFL...........................
P47L/PIG        TLASMLMEENFLRPVGARSTGAIRSGDLVEQVSWMTSRALYTF.................
P47L/MOUSE      TLASMLMEENFLRPVGVRSMGAIRSGDLAEQFLDDST-ALYTFAESYKKKVSSKEEISLS
                ----------------------------------------------======

                                                                    296
                  <-6
P47_HUMAN       KEEFRGVIIKQGCLLKQGHRRKNWKVRKFILREDPAYLHYYDPAGAED-PLGAIHLRGCV
P47/MOUSE       REEFRGVIIKQGCLLKQGXPEKNWKVRKFILREDPAYLHYYDPAGGED-PLGAVHLRGCV
P47L/MOUSE      TMELSGTVVKQGYLSKQGHKRKNWKVRRFVLRKDPAFLHYYDPSKEENRPVGGFSLR...
                      ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++

                                                                  350
P47_HUMAN       VTSVESNSNGRKSEEENLFEIITADEVHYFLQAATPKERTEWIKAIQMASRTGK
P47/MOUSE       VTSVESSHDVKKSDEENLFEIITADEVHYYLQAATSKERTEWIKXIQVGSRTRK
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Fig. 3. Tentative chicken and mouse pleckstrin sequences (P47) are from ESTs with EMBL accession numbers AA495710 and AA543860, respectively. The paralogous pleckstrin-like (P47L) sequences from mouse, human and pig are from ESTs AA543860, AA226122 and SSC1F11. The PH domains are marked by (+), the DEP domain by (−). The numbers followed by an arrow above the alignment indicate the different constructs as summarized in Table 1. Numbers in italics give the positions of the residues in the human pleckstrin sequence. DEP3.4, for instance, spans from M132 to D221 and is indicated as the construct with the N-terminal site 3 and the C-terminal site 4. Positions, identical in pleckstrin and its paralogue are shown in bold. Although preliminary, this analysis indicates that the PH domains are more conserved than DEP. On the other hand, DEP is more conserved than the interdomain regions. The extension of the DEP domain, identified in this work and marked by an equal sign is not conserved between pleckstrin and its paralogues.

newly identified DEP homologue sequences were multiply aligned. This procedure did not reveal significant sequence similarities in C-terminal regions that might have indicated the presence of a secondary structural element additional to those already proposed [15]. A similar conclusion was reached following the alignment of paralogous sequences, such as Dsh homologues. A TBLASTN search [18] of expressed-sequence tag (EST) databases using human pleckstrin as the query sequence was also performed. It revealed partial sequences of chicken and mouse pleckstrin (accession codes AA495710 and AA543860) (Fig. 3). In addition, the search revealed human and mouse sequences (accession codes AA226122 and AA403397) similar to a pig pleckstrin-like sequence identified previously [15]. It is apparent that at least two pleckstrin-like sequences (or 'paralogues') are present in the human genome, each containing a PH–DEP–PH domain architecture. Inspection of an alignment of these sequences (Fig. 3) shows that there is substantial sequence similarity between the paralogues, particularly within the two PH domains and, to a lesser degree, within the DEP domain. However, no convincing similarity is observable within the C-terminal octapeptide (SGFFCEEN) sequence of the two paralogues whose presence is required to stabilize the pleckstrin DEP domain. No obvious frameshifts or nucleotide errors are present in the mouse EST (AA403397) that codes for this region.

Although preliminary, this analysis indicates that the PH domains are more conserved than DEP. On the other hand, DEP is more conserved than the interdomain regions. The extension of the DEP domain, identified in this work and marked by an equal sign is not conserved between pleckstrin and its paralogues.

3. Discussion

The results presented here allow definition of the domain boundaries of the DEP module in pleckstrin. As in other examples widely discussed in the literature, sequence analysis of protein modules provides a first guideline to obtain a stable domain, which needs experimental revision in order to take into account possible variabilities in the motif stability and/or low sensitivity of the biocomputing tools in regions with very low sequence identities [19–21]. In the pleckstrin DEP module, C-terminal extension of the pattern suggested by sequence analysis is necessary in order to obtain a stable domain. Since the sequence is not conserved in other DEP-containing proteins, the C-terminal octapeptide might form a secondary structure element present only in pleckstrins. A similar behaviour has also been described for the WW domain from the kinase associated protein YAP, an autonomously folded domain involved in signal transduction [21]. On the other hand, we cannot at the present stage exclude that unconserved sequences might form a similar secondary structure in other members of the DEP family but remain undetectable by sequence analysis, or that the extension forms a largely unstructured region that, nevertheless, contributes to the shielding of hydrophobic residues from the solvent. Only full structure determination of pleckstrin and of other DEP domains will be able to discriminate between these possibilities.

Thermal stability studies of constructs spanning the DEP domain show that N-terminal extensions lead to gradual destabilization of the DEP fold (Table 1) suggesting that these additional regions are unfolded. The N-terminal extension includes residues S113, T114 and S117 that are thought to provide a multiple phosphorylation site on pleckstrin for PKC [16]. It should be noticed that S117 is fully conserved in all pleckstrin and pleckstrin-like sequences. Our data suggest that phosphorylation might occur in a region with no substantial indications of structure. We speculate that phosphorylation of pleckstrin in this region between the N-terminal PH domain and the DEP module prevents self-association of the protein and therefore regulates interactions of the PH domains with various ligands [22]. Replacement of any of the potentially phosphorylated residues by negatively or positively charged amino acids have an effect on pleckstrin activation similar to that obtained by phosphorylation [16]. According to these results, it appears more likely that it is introduction of a charged group more than phosphorylation itself what enhances pleckstrin activity. Introduction of charges together with steric hindrance imposed by the aliphatic side chains of lysines and glutamates would have a steric effect on the conformation of pleckstrin keeping the active surface of the N-terminal PH domain

far from the DEP domain and therefore available for ligand binding.

4. Materials and methods

4.1. Preparation of the pleckstrin cDNAs

Total RNA from three kind of hematopoietic cell lines (neutrophils C19 and HL60, and eosinophil HL60) was kindly supplied by Kelly McNagny (EMBL). Of the total RNA, 2.5 μg was incubated with 1.5 μl of random hexamer primer 3′ at 95°C and then transferred to ice. The reverse transcriptase reaction (RT) was then performed by the addition of the RT enzyme, dNTPs, RNase inhibitor. The mixture reaction was incubated for 1 h at 37°C. Two microliter of this mixture was used as template in the PCR reaction with the pleckstrin specific primers.

4.2. Cloning and expression of the DEP domain

The DNA sequences corresponding to the DEP domain containing fragments of human pleckstrin were PCR-amplified with engineered *Nco*I and *Bam*HI cloning sites respectively on 5′ and 3′ ends and then cloned into the *Nco*I and *Bam*HI sites of a pHAT2 plasmid vector following the encoded histidine tag MSHHHHHHSM [23]. These constructs were expressed in the *E. coli* strain BL21 (DE3). Cells were grown in LB medium at 37°C and the proteins were induced for 3 h in a 4-l culture by addition of 0.2 mM IPTG after the culture had reached an OD of 0.7 at 600 nm. In each case, expression of the constructs was readily apparent from the increase in amount of a protein of about the expected molecular weight on SDS-PAGE. Identification of all polypeptide chains with respect to sequence was confirmed by mass spectrometry. Purification of the all constructs was carried out following an identical procedure. The pellet was resuspended in 50 ml of 3 mM $MgCl_2$, 0.1 mM PMSF, 50 mM phosphate buffer pH 7.9, 0.1 mg/ml DNaseI. Cells were lysated by French press at 4°C and centrifuged. The soluble protein was purified by Ni-NTA column (Quiagen) chromatography and eluted with a linear gradient of 10 to 200 mM imidazole (pH 7.0) in a standard lysis buffer. The pooled fractions were dialyzed against 50 mM phosphate buffer pH 7.0, 50 mM NaCl and 1 mM DTT and loaded onto a HiLoad 16/60 Superdex 75 column equilibrated in the same buffer.

4.3. Electrophoresis

SDS-PAGE was performed using 15% (w/v) polyacrylamide gels in the Laemmli system. Native PAGE was performed using 15% (w/v) polyacrylamide gels buffered at pH 7.4 with 43 mM imidazole and 35 mM Hepes. The electrophoresis was at 200 V, constant voltage, and at 4°C.

4.4. Circular dichroism

CD experiments were performed on a Jasco J-710 spectropolarimeter equipped with a thermostatically controlled cell holder stabilized by circulating water from a neslab RTE-110 water bath. A rectangular quartz thermostated cuvette with a 0.1 cm path-length was used (Hellma). All the spectra were baseline corrected by subtracting buffer spectra and the observed ellipticity was then converted to mean residue weight ellipticity $[\theta]/(\text{deg cm}^2\ \text{dmol}^{-1})$. The protein concentration was determined by measuring the dilution into 6 M guanidine hydrochloride of a concentrated protein stock solution (1 mM) assuming tyrosine absorbance at 276 nm. An extinction coefficient of 1450 M^{-1} cm and 145 M^{-1} cm as reported for Gly-L-Tyr-Gly at 276 nm and for Gly-L-Cys-Gly at 276 nm respectively was used. The pH was adjusted by diluting aqueous solutions of protein in 50 mM NaH_2PO_4/Na_2HPO_4 buffer.

CD spectra were averaged over five scans obtained by collecting data at 0.5 nm intervals from 250–190 nm. The temperature dependent folding and unfolding were followed monitoring ellipticity at 215 nm from 10 to 90°C at a heating rate 20°C/h.

4.5. NMR measurements

The NMR experiments were performed on a Bruker AMX-500 and on an AMX-600 spectrometer equipped with *z*-shielded gradient coils using 1.0–1.5 mM samples in 90% H_2O/10% D_2O at pH 6.1–7.0, 10 mM NaCl and 5 mM DTT.

4.6. Sequence analysis

Sequence analyses of DEP domains were performed using techniques used previously [15]. The

TBLASTN program [18] was used to retrieve potential pleckstrin paralogues and/or orthologues from ESTs. Possible double frameshifts were checked using the Wisetools program [24].

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