Chapter 2

Structure and PtdIns(3,4)P₂ binding of pleckstrin C-PH

Pleckstrin is a protein of three domains: the prototypic PH domains at each terminus (N-PH and C-PH), separated by the DEP domain and two inter-domain linkers. The structures of N-PH and DEP are known (Yoon et al., 1994;Civera et al., 2004), but not the structure of C-PH. In fact, C-PH is structurally and biochemically totally uncharacterised. In the present chapter, the NMR structure determination and phospholipid binding properties of C-PH are described. This does not only serve to elucidate the biological function of pleckstrin C-PH, but also to obtain the last building block that is required to assemble the structure of the full-length protein, as described in the following chapters.

2.1 Results

2.1.1 Construct definition



Figure 2.1: Linker sequence between DEP and C-PH domains. The last and the first β -strand of DEP and C-PH, respectively, are represented as grey boxes. Hydrophobic residues in the linker are in white on a black background. Arrows indicate the N-termini of the four constructs of C-PH.

The first step in structure determination is to define an optimal construct. The C-PH domain of human pleckstrin starts with amino acid 246 and end s with 347 in the

SMART alignment (www.smart.embl.de). The latter boundary practically coincides with the native C-terminus (aa 350). The N-terminal boundary is preceded by 20 amino acids of the DEP_C-PH linker sequence which contains several hydrophobic residues. To test whether any of these may be required for a stable and folded sample, four N-terminally extended C-PH constructs (Fig. 2.1) are cloned, expressed and checked by NMR (1 H, 15 N-HSQC). The longest one starting at the boundary of the DEP domain (C-PH₂₂₁₋₃₅₀) is degraded in *E.coli*. The shortest one with just four residues more than the sequence alignment (C-PH₂₄₂₋₃₅₀) gives good spectra and expression yields; however, it has a strong tendency to aggregate. Both intermediate constructs (CPH₂₃₄₋₃₅₀ and C-PH₂₃₈₋₃₅₀) are folded and more stable. From preliminary analysis of the DEP_C-PH double-domain construct (chapter 3), it is known that the three central aspartate residues of the linker (232-234) are completely disordered and may be considered the true "divide" between the domains. Thus, C-PH₂₃₄₋₃₅₀ containing the additional three hydrophobic residues Val²³⁵, Ile²³⁶ and Leu²³⁷ is selected for structure determination and all subsequent experiments.

2.1.2 Resonance assignment of C-PH

Backbone resonance assignment is achieved by means of standard triple-resonance experiments (see Chapter 6, "Materials and Methods"). Two regions of the polypeptide chain cause problems during the assignment procedure: (i) the $\beta 1-\beta 2$ loop (His²⁵⁶-Arg²⁵⁸), for which no H^N/N resonances can be assigned and (ii) Asn³⁰⁵-Gly³⁰⁶ in the $\beta 5-\beta 6$ loop that each give rise to two extremely different H^N/N resonance pairs. The missing assignments for the $\beta 1-\beta 2$ loop are not entirely unexpected because this region is also unassigned in several PH domains, including pleckstrin N-PH (Yoon et al., 1994). Probably, this loop experiences conformational exchange on intermediate time-scales (see below). Conversely, the doubling of Asn³⁰⁵-Gly³⁰⁶ is caused by a rarely described isomerisation of the peptide bond: Asn³⁰⁵ is deaminated to iso-aspartate and the peptide bond to Gly³⁰⁶ is formed through the sidechain carbonyl (C γ , Fig. 2.2A). The atypical peptide bond can be identified in most triple-resonance experiments because the C α and C β positions are swapped with respect to Gly³⁰⁶ (C β now being next to the peptide carbonyl). The deamination of Asn³⁰⁵ appears to be spontaneous and reaches a stable

level of about 50%, as judged from peak intensities of Asn³⁰⁵ and iso-Asp³⁰⁵. An assigned ¹H,¹⁵N-HSQC is shown in Fig. 2.2B and the two peaks for Ans³⁰⁵ and iso-Asp³⁰⁵ are highlighted in red.

Almost complete sidechain resonance assignment is obtained in the course of iterative steps of spectral assignment and structure calculation. Interestingly, the resonances of two exchangeable and scarcely observable protons can be assigned: the H γ 1 (hydroxyl) of Thr³¹⁹ which participates in a hydrogen bond network in the short loop connecting β 6 and β 7, and H γ (SH) of Cys²⁹⁵ which resides in the hydrophobic core (and which is not accessible to cysteine-specific spin-label reagents, chapter 4).



Figure 2.2: Assigned ¹H, ¹⁵N-HSQC of C-PH. A: The deamination reaction of asparagine to iso-aspartate. B: Assigned ¹H, ¹⁵N-HSQC of C-PH. Sidechain resonances are indicated with a Greek letter. The two sets of signals for Asn³⁰⁵-Arg³⁰⁷ are highlighted in green (native) and red (iso-Asp³⁰⁵).

2.1.3 Structure and dynamics of pleckstrin C-PH

The structure of C-PH is calculated using NOE, dihedral angle and RDC restraints as input for a simulated annealing protocol (Linge et al., 2001). The statistics of the structure determination are summarised in Table 1. Structural quality and precision are satisfactory for the final ensemble of structures, and very good for the structured part of the domain (excluding flexible termini and loops), which is also evident from the superposition of the NMR ensemble in Fig. 2.3A: the backbone traces of the ten lowest energy structures superimpose very neatly over all secondary structure elements (red and blue in Fig. 2.3A).



Figure 2.3: Structure of pleckstrin C-PH. A: Superposition of the 10 best structures (out of 80 calculated) after water refinement. α -helices are in red and β -sheets in blue. Termini and most prominent loops are labelled. **B**: Ribbons representation. Colouring and labelling as for panel **A**.

C-PH has the typical PH domain fold – a seven-stranded β -barrel capped at one side by the C-terminal helix (α 2) - with one additional secondary structure element, a one-turn N-terminal α -helix (α 1, aa 238-242). The aromatic sidechain of Phe²⁴¹ packs against the first β -sheet of C-PH explaining why constructs that include this residue are more stable. The N-terminal residues 234-237 are disordered and not in contact with the core of the domain. Thus, with hindsight, it is likely that C-PH₂₃₈₋₃₅₀ would have been the optimal C-PH construct.

The front view of Fig. 2.3 shows the β -barrel with the C-terminal helix (α 2) at the bottom. Two loops (β 1- β 2 and β 3- β 4) form the opposite "open" side. In the top view, it is evident that this side of the barrel is indeed quite accessible. Both views are also shown in ribbon representation (Fig. 2.3B).

A database search for similar structures is carried out because structural similarity often suggests a comparable function of a protein. A comparison of the structure of C-PH with all structures in the protein data bank (PDB) reveals that C-PH most strongly resembles a sub-class of PH domain structures. The MSD-fold search algorithm (www.ebi.ac.uk/msd-srv/ssm/) ranks the structures of the PH domains of DAPP1, TAPP1 and PKB as the most similar structures in the PDB. Except for minor differences in loop lengths, the backbone traces of these PH domains are completely superimposable with C-PH. Since the PH domains of DAPP1, TAPP1 and PKB are all highly specific phosphoinositide binders (Dowler et al., 2000; Frech and Hemmings, 1998; Kavran et al., 1998, cf. chapter 1) this finding raises the possibility that C-PH could also have a function in phosphoinositide binding.

Table 1: Structural statistics of the pleckstrin C-PH domain¹.

	<sa>²</sa>	<sa<sup>watref>²</sa<sup>
R.m.s. deviation (Å) from experimental distance restraints ³		
Unambiguous/ambiguous (3176/115)	0.02320 ± 0.0007	0.02758 ± 0.0007
R.m.s. deviation (°) from experimental torsion restraints ⁴		
Dihedral angles (65 ϕ , 65 ψ)	0.24 ± 0.04	0.17 ± 0.05
Quality factor for RDCs ⁵		
H ^N -N (33)	0.074 ± 0.007	0.116 ± 0.009
Coordinate Precision (Å) ⁶		
Ν, C ^α , C'	0.35 ± 0.07	0.40 ± 0.07
All heavy atoms	0.90 ± 0.07	0.98 ± 0.07
Structural quality ⁷		
Bad contacts	1.2 ± 1.1	0.1 ± 0.3
Ramachandran plot		
% in most favoured region % in additionally allowed region	82.8 ± 1.1 14.9 ± 1.6	86.7 ± 1.9 11.4 ± 2.0

¹ Structured part excluding N- and C-termini (aa 234-237 and 347-350) and two unstructured loops (aa 255-260, 281-293)

 2 <SA> is an ensemble of 10 lowest-energy solution structures of the pleckstrin C-PH domain (out of 80 calculated). The CNS E_{repel} function is used to simulate van der Waals interactions with an energy constant of 25.0 kcal mol⁻¹ Å⁻⁴ using "PROLSQ" van der Waals radii; R.m.s. deviations for bond lengths, bond angles and improper dihedral angles are 0.00216 ± 0.00007 Å, 0.407 ± 0.006° and 0.384 ± 0.014°. 1 kcal = 4.18 kJ. For <SA^{watref}>, the ensemble of <SA> structures is refined in a shell of water as described (Linge et al., 2003).

³ Distance restraints are employed with a soft square-well potential using an energy constant of 50 kcal mol⁻¹ Å². No distance restraint is violated by more than 0.3 Å in any of the final <SA> structures.

⁴ Dihedral angle restraints, derived from an ³J(H^N,Hα) coupling constants and from TALOS (Cornilescu et al., 1999) are applied to ϕ , ψ using energy constants of 200 kcal mol⁻¹ rad⁻². No dihedral angle restraint is violated by more than 5°.

⁵ Backbone H^N-N residual dipolar couplings are applied with a final energy constant of 0.3 kcal mol⁻¹ Hz⁻² for an alignment tensor with an axial component of 13.5 Hz and a rhombicity of 0.55. The two Trp H ϵ 1-N ϵ 1 RDCs are treated separately and applied with a final force constant of 1.0 kcal mol⁻¹ Hz⁻². The quality factor Q for the backbone H^N-N RDCs is calculated as described (Cornilescu et al., 1998).

⁶ Coordinate precision is given as the Cartesian coordinate r.m.s. deviation of the 10 lowest-energy structures with respect to their mean structure.

⁷ Structural quality is analyzed using PROCHECK (Laskowski et al., 1996).

The analysis of ¹⁵N relaxation data is a valuable tool to characterise backbone dynamics of a protein. The results of R1, R2 and heteronuclear NOE experiments are shown in Fig. 2.4. The low heteronuclear NOE and R2/R1 values of residues 234 to 237 demonstrate that the N-terminus up to residue 237 is flexible. By contrast, residues 238-242 (helix α 1) have higher heteronuclear NOE values consistent with a structured element, although the values are slightly lower than for the core of the PH domain. The two poorly structured loops of C-PH (β 1– β 2 and β 5– β 6, Fig. 2.3) both experience backbone dynamics. The resonances of the former (His²⁵⁶-Arg²⁵⁸) are not observed, however, the flanking residues (Gly²⁵⁵ and Lys²⁵⁹) display elevated R2/R1 ratios, indicating dynamics on the milli- to microsecond time-scale. This suggests that the entire β 1– β 2 loop experiences conformational exchange on intermediate timescales. By contrast, the loop β 5– β 6 (encompassing residues Ser³⁰¹-Asn³¹³) is characterised by low heteronuclear NOE and R2/R1 values which indicate fast dynamics on pico- to nanosecond time-scales, demonstrating that it is truly flexible. The spontaneous deamination of Asn³⁰⁵ to isoaspartate is probably favoured by the completely unstructured nature of this loop.

The overall correlation time of 11.5ns estimated from the average R2/R1 value is very high for a domain of 12kDa at 22°C. This indicates that C-PH experiences partial and transient dimerisation or aggregation. The relaxation data are recorded at a protein concentration of 0.5mM, which is not very high, considering that other PH domains only start to show aggregating tendencies above 1mM concentrations (Fushman et al., 1997; Gryk et al., 1998). For the PH domain of β -spectrin, it is shown that the aggregation tendencies do not involve any particular regions of the domain, but are a global and unspecific phenomenon (Gryk et al., 1998). This is probably also the case for C-PH and it explains the tendency of the protein to precipitate at preparative stages and after longer periods at ambient temperatures. Unfortunately, a more detailed analysis of the dynamics of C-PH is not possible: all approaches fail if a protein does not behave like a pure monomer in solution (Schurr et al., 1994).



Figure 2.4: NMR relaxation parameters of C-PH. A: {¹H}-¹⁵N heteronuclear NOE. B: R2/R1 ratio.

2.1.4 Protein lipid overlay assay

Sequence and structure of C-PH strongly suggest that C-PH could have a role in phosphoinositide binding. First, C-PH contains a sequence motif that is conserved in many phosphoinositide binding PH domains (Isakoff et al., 1998 cf. section 2.1.6). Second the structural similarity to DAPP1, TAPP1 and PKB points into the same direction. The standard procedure to identify phosphoinositide binding by PH and similar domains is the protein lipid overlay assay (Dowler et al., 2002). In this assay, a GST-tagged PH domain is allowed to

bind to a membrane onto which different lipids have been spotted. After several wash steps, bound protein is detected with an antibody to GST.



Figure 2.5: Protein lipid overlay assays with GST-CPH fusion protein. In panel A and B, C-PH is used as a probe. Panel C shows the positive and negative controls (PLC- δ is specific for PtdIns(4,5)₂, TAPP1 is specific for PtdIns(3,4)₂ and GST is the negative control). The amount of lipid per spot is 200 pmoles in panel A and as indicated in panel B and C. Protein concentrations are 1µg/ml C-PH, 0.1µg/ml PLC- δ and TAPP1, 5µg/ml GST. Abbreviations for the lipids: S1P: sphingosine-1-phosphate, LPA: lysophosphatidic acid, LPC: lysophosphocholine, PE: phosphatidylethanolamine, PS: phosphatidylserine, PA: phosphatidic acid, PC: phosphatidylcholine, PtdIns: phosphatidylinositol.

The assay is carried out with GST-C-PH and GST fusion proteins of the PH domains of PLC- δ and TAPP1 (positive controls) and GST alone (negative control). As shown in Fig. 2.5A, C-PH only binds strongly to PtdIns(3,4)P₂. This is a quite an extraordinary result because merely two other PH domains, TAPP1 and the homologous TAPP2, have been described that have a similar specificity (Dowler et al., 2000). To corroborate this surprising finding, the assay is repeated with membranes where phosphoinositides are spotted in decreasing amounts (Fig. 2.5B). Again, C-PH only binds PtdIns(3,4)P₂. Even low amounts of this phosphoinositide are detected. GST alone shows no signal at all and the positive controls

give the expected binding pattern (PLC- δ for PtdIns(4,5)P₂ and TAPP1 for PtdIns(3,4)P₂, (Dowler et al., 2000; Lemmon et al., 1995) Fig. 2.5C). However, smaller amounts of PLC- δ and TAPP1 are needed to elicit the same signal intensity compared to C-PH (Fig. 2.5B). Since the same antibody is used for detection, this indicates that C-PH has a relatively lower affinity for its ligand than do both PLC- δ and TAPP1. How much lower the affinity is cannot be judged from this assay because GST is a dimer and the kinetic effects of the wash steps are difficult to account for. In any case, pleckstrin C-PH is a highly specific PtdIns(3,4)P₂ binder with a somewhat lower affinity than the PH domains of TAPP1 and PLC- δ .

2.1.5 NMR titrations with $Ins(1,3,4)P_3$

Chemical shift is a sensitive probe of local environment. Therefore it is an excellent tool to monitor changes induced by ligand binding. Unlike the protein lipid overlay assay, NMR titration experiments (i.e. stepwise addition of ligand to a protein sample) measure the interaction between protein and ligand directly by monitoring chemical shift. Moreover, the results allow mapping of the ligand binding site on the protein.

When the soluble headgroup of PtdIns(3,4)P₂, Ins(1,3,4)P₃, is added to a sample of C-PH the ¹H,¹⁵N HSQC spectrum changes notably (Fig. 2.6A). Saturation is already reached at a 2:1 excess of ligand and many NMR signals (peaks) experience large chemical shift changes. The most strongly affected peaks are in intermediate exchange and cannot be traced reliably, hence the assignments of the bound state is obtained by analysing a ¹⁵N-HSQC NOESY of a sample with a 4:1 molar excess of ligand. Remarkably, the resonances of the $\beta 1-\beta 2$ loop (Gly²⁵⁵-Lys²⁵⁹) become visible and can be assigned - in contrast to the ligand-free state. This suggests that the $\beta 1-\beta 2$ loop becomes ordered in the ligand-bound state. Moreover, an unusual ¹H-¹⁵N resonance pair appears at 9.55/88.7 ppm that may belong to an arginine H^e/N^e group. Since the ¹H chemical shift is very unusual for an arginine sidechain group it could mean that it is in a salt bridge to the ligand.



Figure 2.6: NMR titrations with $Ins(1,3,4)P_3$. A: The ¹H, ¹⁵N-HSQC spectrum of free C-PH (black) is overlaid with a spectrum where a 4:1 excess of $Ins(1,3,4)P_3$ has been added (red). The most strongly shifting peaks are labelled with their residue number. **B**: chemical shift difference ($\Delta \delta$) of backbone amide groups between free and bound forms. Results are shown for titrations with $Ins(1,3,4)P_3$ (red) and $Ins(1,3,4,5)P_4$ (green).

In order to localise the binding site on C-PH, the resonance assignments of free and bound form of C-PH are compared and the difference in chemical shift ($\Delta\delta$) is calculated. In Fig. 2.6B, the $\Delta\delta$ value of each backbone group is plotted against its sequence position. The largest $\Delta\delta$ values of backbone amide groups map to the β 1, β 2 and β 7 strands (Fig. 2.6B). The most strongly affected sidechain ¹H-¹⁵N groups also belong to these β -strands (data not shown). Thus, the binding site consists of residues in the β 1– β 2 loop and the β 1, β 2 and β 7 strands.

Since all described PH domains that bind $Ins(1,3,4)P_3$ also bind $Ins(1,3,4,5)P_4$ to some degree (Lemmon, 2003), the NMR titration experiments are repeated with $Ins(1,3,4,5)P_4$ – the NMR experiment is more sensitive to weak interactions than protein lipid overlay assays. Only small chemical shift changes are observed for $Ins(1,3,4,5)P_4$ (green in Fig. 2.6B). A molar excess of 10:1 is needed to reach saturation, the peaks are in fast exchange and the resonances of the $\beta 1$ – $\beta 2$ loop residues (Gly²⁵⁵-Lys²⁵⁹) do not become observable. All of this shows that the interaction with $Ins(1,3,4,5)P_4$ is much weaker than with $Ins(1,3,4)P_3$, which is in agreement with the results of the protein lipid overlay assay (Fig. 2.5). In the regime of fast exchange, the dissociation constant for the interaction (K_D) can be calculated from the NMR titration data. For $Ins(1,3,4,5)P_4$ the K_D equals $30\pm15\mu$ M. The K_D for the interaction with $Ins(1,3,4)P_3$ cannot be calculated directly because the peaks are in intermediate exchange. However, by comparison with the $Ins(1,3,4,5)P_4$ titration data, it is estimated to be about one order of magnitude lower, i.e. in the low micromolar range.

2.1.6 Comparison with known PH domain: $Ins(1,3,4,5)P_4$ complex structures Several structures of PH domains in complex with $Ins(1,3,4,5)P_4$ have been solved (Baraldi et al., 1999; Ferguson et al., 2000; Lietzke et al., 2000; Thomas et al., 2002), but none in complex to $Ins(1,3,4)P_3$. Two of the complex structures (PKB and DAPP1) are solved for PH domains that bind $Ins(1,3,4,5)P_4$ and $Ins(1,3,4)P_3$ equally well (Ferguson et al., 2000; Thomas et al., 2002). Their structures are very similar to C-PH, therefore the ligand contacts in the crystal structure can be compared to C-PH residues that are strongly affected in NMR titration experiment with $Ins(1,3,4)P_3$ ($\Delta\delta$ >0.2, Fig. 2.4B). Although its structure has only been solved in complex to a citrate molecule, TAPP1 is also included in the analysis because it is the only known $Ins(1,3,4)P_3$ specific PH domain (Thomas et al., 2001). Finally, the two exclusively $Ins(1,3,4,5)P_4$ binding PH domains of Grp1 and Btk for which complex structures are available (Baraldi et al., 1999; Ferguson et al., 2000; Lietzke et al., 2000) are also shown in the Fig. 2.7.

The ligand contacts of all crystal structures fall into two categories: a) the "clamp" motif (Isakoff et al., 1998) consisting of KxG on β 1, N/TxKxR on β 2 and Y β 3 and b) contacts from the "specificity determining regions" (SDRs Lietzke et al., 2000), i.e. from the β 1– β 2, β 3– β 4, β 6– β 7 loops and the β 7 strand. The affected residues of C-PH may be categorised in the same way: a) the primary binding site around the motif (Lys²⁵³, Gln²⁵⁴, Trp²⁶¹, Lys²⁶², Arg²⁶⁴) and b) additional contacts from the β 7 strand and the β 1– β 2 loop. There are also subtle differences to the known structures. In particular, there are no strongly affected C-PH residues on strands β 3 and β 4. However, the comparison shows that, by and large, the ligand contacts are very similar. Thus, the mode of binding of C-PH to Ins(1,3,4)P₃ will resemble the known complex crystal structures.



Figure 2.7: Structure based sequence alignment with high affinity PH domains. The sequence motif that is required for binding to D3 phosphorylated phosphoinositides is highlighted with black bars. Additional residues that make ligand contacts in crystal structures are in bold type and boxed. Residues of C-PH that have a $\Delta \delta$ >0.2 (strongly affected by $Ins(1,3,4)P_3$ binding) are marked with an asterisk. $\beta I - \beta 2$ loop residues that are only assigned in the bound form are marked with \ddagger . The secondary structure elements of C-PH are given above the alignment.

The results from the NMR titrations with $Ins(1,3,4)P_3$ can also be used to map the binding site on the structure of C-PH. In Fig. 2.8A, the residues of C-PH are colour coded

according to their chemical shift difference ($\Delta\delta$) between free and Ins(1,3,4)P₃-bound form: strongly affected (red), intermediately and weakly affected (orange and yellow), unaffected (blue) and no data available (prolines, grey). Hence, the binding site is highlighted by red and orange colouring. It is located at the "open" side of the domain. The electrostatic potential of C-PH (Fig. 2.8B) shows a cluster of positive charges in the vicinity of the binding pocket. Basic residues from $\beta 1$ (Lys²⁵³) and $\beta 2$ (Lys²⁶², Arg²⁶⁴) as well as from the $\beta 1$ - $\beta 2$ loop (Arg²⁵⁷, Arg²⁵⁸ and Lys²⁵⁹) contribute to the positive potential. Thus, the Ins(1,3,4)P₃ binding site comprises basic residues from the conserved motif and additional charges from the $\beta 1$ - $\beta 2$ loop.



Figure 2.8: Mapping of the $Ins(1,3,4)P_3$ binding site of C-PH. A: Difference in chemical shift ($\Delta\delta$) between free and bound form of C-PH is colour coded, ranging from red (strongly affected) over orange and yellow (intermediately affected) to blue (unaffected). Grey: no data (prolines). B: Electrostatic potential of C-PH at $\pm 10 k_BT$ in surface representation. The structure is rotated 90° (top view) with respect to panel A.

2.2 Discussion

It has been known for a long time that pleckstrin is the major PKC substrate in platelets. Yet, its role in the platelet signalling network is poorly understood. Especially the function of the C-terminal PH domain remained mysterious. The work presented in this chapter shows that C-PH is a highly selective $PtdIns(3,4)P_2$ binder. This is consistent with a report claiming that both C-PH and full length pleckstrin bind to large unilamellar vesicles containing $PtdIns(3,4)P_2$ (Sloan et al., 2002).

The data presented in this chapter also show that the binding site of C-PH is located at the "open" side of the domain. The basic residues from strands $\beta 1$ and $\beta 2$ probably capture the D3 and D4 phosphates of PtdIns(3,4)P₂ in a manner similar to the known crystal complex structures. There are also large values of $\Delta \delta$ on the $\beta 7$ strand. It is possible that they originate from a structural rearrangement of the $\beta 1$ strand that is transmitted to the $\beta 7$ strand through the two hydrogen bonds between them. Another possibility is that a residue from $\beta 7$ contributes some ligand contacts. The only candidate for this role is Tyr³²⁵: its sidechain points into the direction of the ligand whereas all other sidechains from $\beta 7$ are too far away. By the same token it is unlikely that the basic sidechains from the $\beta 1-\beta 2$ loop interact with the ligand. However, their contribution to the electrostatic potential of the binding site may be important. Alternatively, the positive charges might become important when binding to the negatively charged inner plasma membrane surface.

But what determines the specificity for PtdIns(3,4)P₂? Most likely it is the $\beta 1-\beta 2$ loop. Rather than providing additional contacts it may sterically exclude binding to other - in particular D5 phosphorylated – inositides, as demonstrated for the TAPP1 PH domain: a single mutation converts TAPP1 into the DAPP1 phenotype and vice versa (Thomas et al., 2001). The critical residue is the one immediately following the KxG half-motif. It is a glycine in DAPP1 (Gly¹⁷⁶), an alanine in TAPP1 (Ala²⁰³) and a histidine in C-PH (His²⁵⁶). Only a glycine in this position seems to allow sufficient conformational freedom to allocate the D5-phosphate of PtdIns(3,4,5)P₃. Thus, the basis of selecting for PtdIns(3,4)P₂ could be the same for TAPP1 and C-PH.

In order to summarise and to visualise all putative ligand interactions, the binding site of C-PH is shown in an overlay with the DAPP1:Ins(1,3,4,5)P₃ complex (Fig. 2.9). The sidechains of Lys²⁵³, Lys²⁶², Arg²⁶⁴ and Tyr³²⁵ are highlighted. Consistent with their proposed role in ligand coordination, they are near the ligand molecule. The β 1– β 2 loop has a conformation that would exclude binding of D5 phosphorylated inositides.



Figure 2.9: Details of the binding pocket. C-PH is overlaid with the DAPP1:Ins $(1,3,4,5)P_4$ complex structure. C-PH is in grey and DAPP1 in blue. The inositol moiety of the ligand is drawn in black. Phosphate groups are represented as spheres, phosphorous and oxygen atoms are in green and red, respectively. The sidechains of C-PH residues which are thought to participate in binding to $Ins(1,3,4)P_3$ are also shown and labelled. In this orientation, the $\beta 1 - \beta 2$ loop of C-PH clashes with the D5 phosphate.

It is interesting to note that the $\beta 1-\beta 2$ loop seems to play a very important role for many PH domains in discriminating between different phosphoinositides. Although there are no hard and fast rules to predict the specificity of a (unknown) domain, there are certain tendencies: PH domains with a preference for PtdIns(3,4,5)P₃ often have longer $\beta 1-\beta 2$ loops (>10 residues), whereas domains that bind PtdIns(3,4)P₂ have shorter ones (6 residues). This basic specificity gets altered by the number of glycine residues that follow the KxG half motif in shorter loops. While a second glycine seems to be optimal for PtdIns(3,4,5)P₃, a third one changes the specificity again: a splice variant of ARNO (homologous to Grp1 and also specific for PtdIns(3,4,5)P₃) with an inserted third glycine binds both PtdIns(3,4,5)P₃ and PtdIns(4,5)P₂ with lower affinity (Klarlund et al., 2000).

What are the biological implications of the high specificity of C-PH for PtdIns $(3,4)_2$? Is the observed binding relevant in vivo? It has been reported that C-PH does not to translocate to the membrane when transfected into several cell types (Ma and Abrams, 1999; Ma et al., 1997). However, C-PH may still have a function in membrane targeting or association. The transfection experiments only show that C-PH is localised in the cytoplasmic compartment of unstimulated cells. If cells are stimulated in order to produce the PtdIns $(3,4)P_2$ the situation may be different. Unfortunately there are no such data on C-PH of pleckstrin-1 *in vivo*, but there are for pleckstrin-2 (80% homology for C-PH). In a yeast rescue assay that depends on targeting to the plasma membrane in response to the products of PI-3K (i.e. PtdIns $(3,4,5)P_3$ and PtdIns $(3,4)P_2$), C-PH of pleckstrin-2 from (EST230143) gives a positive result as do TAPP1, DAPP1 and many others (Isakoff et al., 1998). The sequence differences between C-PH of pleckstrin-1 and pleckstrin-2 are minor and not in the specificity determining regions. Therefore C-PH of pleckstrin-1 may very well be a PH domain capable of membrane targeting in response to $PtdIns(3,4)P_2$.

In stimulated platelets, an initial burst of PtdIns(3,4,5)P₃ synthesis is followed by a slower rise in PtdIns(3,4)P₂ (Toker et al., 1995). The latter depends on a synthesis pathway that is independent of the dephosphorylation of PtdIns(3,4,5)P₃ and has only been described in platelets (Banfic et al., 1998). Interestingly, there are also two distinct phases of pleckstrin phosphorylation (Toker et al., 1995). The initial phosphorylation parallels the PtdIns(3,4,5)P₃ peak but is not wortmannin (a PI-3K inhibitor) sensitive, whereas the second phase of sustained phosphorylation is. Addition of PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ also directly stimulates pleckstrin phosphorylation. Since some atypical PKCs can be activated with PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ (Toker et al., 1994), the authors conclude that the sustained phase of pleckstrin phosphorylation depends to a large extent on stimulation of atypical PKCs by PtdIns(3,4)P₂ (Toker et al., 1995).

In the light of our results it is likely that pleckstrin responds to the elevation of $PtdIns(3,4)P_2$ directly through its C-terminal domain. The correlation between $PtdIns(3,4)P_2$ levels and sustained pleckstrin phosphorylation may depend on C-PH in several ways. On the one hand, membrane localisation mediated by C-PH may stimulate phosphorylation or inhibit dephosphorylation. On the other hand, pleckstrin's translocation to the membrane in response to $PtdIns(3,4)P_2$ could in itself trigger the activation of signalling pathways that in turn activate a subset of PKCs. The situation is very complicated especially because phosphorylation of pleckstrin also results in translocation to the membrane mediated by N-PH. The two PH domains of pleckstrin could work in concert or at different points in time or space. How phosphorylation Intramolecular interactions, as well as yet unidentified protein ligands, may additionally affect the behaviour of all components of the molecule *in vivo*. There is still much to learn, however, the structure and characterization of C-PH provides an important step towards the understanding of the pleckstrin protein.

2.3 References

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