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RESEARCH ARTICLE

# Aromatic amino acids and their relevance in the specificity of the PH domain

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## Abstract

Phosphoinositides are phosphatidylinositol derived, well known to be second messengers in various cell signaling pathways as well as in processes such as cell differentiation, cellular stress response, gene transcription, and chromatin remodeling. The pleckstrin homology domain of phospholipase C-delta 1 is responsible for recognizing and binding to $PI(4,5)P_2$ and for this reason has been widely used to study this phosphoinositide as a biosensor when it is conjugated to a fluorescent tag. In this work, we modified the primary structure of pleckstrin homology domain by site-specific mutagenesis to change the specificity for phosphoinositides. We obtained 3 mutants: K30A, W36F, and W36Y with different specificity to phosphoinositides. Mutant domain K30A recognized $PI(4,5)P_2$, $PI(3,4,5)P_3$, phosphatidic acid (PA), and weakly $PI(3,5)P_2$. Mutant domain W36F recognized all the phosphoinositides studied and the PA. Finally, mutant domain W36Y seemed to interact with PA and all the other phosphoinositides studied, except PI(3)P. The changes in recognition argue against a simple charge and nonpolar region model for these interactions and more in favor of a specific docking region with a specific recognition site. We conducted in silico modeling that explains the mechanisms behind the observed changes and showed that aromatic amino acids appear to play more important role, than previously thought, in the specificity of phospholipids' binding domains.

**KEYWORDS**

PH domain, phosphatidic acid, phosphoinositides

## 1 | INTRODUCTION

The use of biosensors is a powerful tool for the study of particular molecules in cells, because they are capable of recognizing specific ligands in vivo.[1]

Biosensors are used to detect phospholipids (PIPs), which derive from phosphatidylinositol (PI). Phospholipids are involved in many signal transduction events as well as in a stress response, cytoskeletal dynamics, cell migration and polarity, vesicular trafficking, pre-mRNA maturation, gene expression, and chromatin remodeling.[2-5] Biosensors are usually the domains of particular proteins, which are capable to recognize the lipid polar heads. This interaction is thought to occur due to the high density of negative charges of the phosphate ($PO_4^{3-}$) groups with compatible charges of the protein domains.[6]

  

The most common domains used to recognize PIPs are the Fab 1, YOTB, Vac 1, and EEA1 zinc finger domain; the four-point-one, ezrin, radixin, moesin domain plant homeodomain; pox domain; and the post-synaptic density protein-95/disks large/zonula occludens-1 domains. Common characteristics of all the domains are the presence of positively charged amino acids and at least 1 aromatic amino acid, which is usually tyrosine or histidine.[7] Some of the domains share a similar fold, but the PIPs they recognize are different. It has been suggest that domains with little affinity and/or specificity for PIPs have steric effects that allow different PIPs to bind. Many of these domains are identified in proteins related to cell signaling, cytoskeletal remodeling, vesicular trafficking, and cell proliferation and death, such as protein kinase B, phosphatase and tensin homolog, phospholipase D, phospholipase C, caspase 3, inhibitor of growth protein, and trithorax-homolog protein 1ATX1.[8,9] The most studied and the most currently used PIP biosensor is the pleckstrin homology (PH) domain of the enzyme phospholipase C-delta 1 (PLCδ1).

Pleckstrin homology domain was originally described as a novel protein motif of about 100 amino acids, repeated twice in the Pleckstrin protein, the main substrate of protein kinase C.[10,11] This domain has been identified in more than 100 proteins, many of them are involved in the signal transduction processes[12,13] through the PH domain interaction with PIPs.

The basic structure of the PH domain is a β sandwich composing of 2 β sheets with 7 β strands (β1-β7), forming an angle of almost 90°. β1 and β2 strands are joined by a loop called VL1, the strands β3 and β4 are connected by the loop VL2, and β6 and β7 strands are linked by the loop VL3. VL1 and VL2 loops are responsible for ligand binding, and the C-terminus has the amphipathic alpha helix.[14,15]

Pleckstrin homology domains show remarkable variations in their sequences, affinities, and specificities, which is why many attempts to classify them according to their sequences were inconclusive. Currently, the most widely accepted classification of PH domains is based on their degree of specificity.[16]

The PLCδ1 PH domain is characterized by the presence of 2 additional α-helices: in the N-terminus and in the loop β5-β6; none of them have a direct contact with the ligand.[17] The PH domain of PLCδ1 binds both to $IP_3$ and $PI(4,5)P_2$ with high affinity,[18,19] while the conformation of short α-helix (residues 82 to 87) can influence the affinity of the PLCδ1 PH domain for $IP_3$.

PIPs recognition sites contain residues with positive charges, which are responsible for interacting with the negative charges of the phosphate groups of PIPs.[7,20] However, although serine and aromatic residues constitute these binding sites, their importance for the specificity of PIPs-binding domains remains poorly understood or ignored.

There are several drawbacks of the use of biosensors, in particular related to their capability to bind more than one specific target as well as the intrinsic background from nonspecific binding. For example, PH domain from Akt1 is widely used as a biosensor for both PI(3,4)P2 and PI(3,4,5)P3, but it has different affinity to these PIPs.[21] Besides, one should consider the differences in the background interactions between various domains. The best way to diminish this effect is to change the affinity of particular biosensors to a given PIP or the affinity of a given biosensor to PIPs.[22]

In this study, we are focused on the understanding of the interactions of different PIPs with the PLCδ1 PH domain. We chose this approach to eliminate the possible effects of different backgrounds from various domains as well as to be able to alter specific targets by particular mutations. We have used the computational modeling of the PH domain with the altered structure of the pocket, which is responsible for the interactions between PIPs and the PH domain. The structural changes of the pocket result in the altered specificity of the PH domain to the various PIPs, while the basic charges, which interact with the phosphates groups of the lipid heads, are unchanged.

## 2 | MATERIALS AND METHODS

### 2.1 | PH domain alignment

Ten different PH domains amino acid sequences alignment was performed in MEGA 6 platform. Followings PH domain sequences registered in NCBI data base were aligned using MUSCLE (multiple sequence alignment by log-expectation) using its default parameters, looking for conserved residues. The sequences used are: gi|1943485, gi|14249340, gi|19115964, gi|1079040, ref/XP 859460.1, gi|417490, ref/AAA93481.2, gb|ACA05825.1, gi|730329, gi|54539.

### 2.2 | In silico mutation and analysis

We used the structure of the PH domain deposited in the Protein Data Bank with the PBD ID 1IMA.[17] In silico single point mutations in PH-domain binding site residues K30, W36 and R40 were made using SWISS PDB Viewer mutation tool. For the mutant domain K30A construction, we selected the residue K30; after this, we choose the mutate tool in the menu, and we choose an alanine for which we replace it. Later, we applied energy minimization calculation using GROMOS 96 software (that is in the SWISS PDB viewer) for the optimal amino acid orientation. After these, only the 6 AA, which conforms the binding site (K30, K32, W36, R40, E54, and S55), was selected using the control panel, and the rest was deleted using the build menu, and the file was stored in PDB format. The same was made for the mutants W36Y, W36F, and R40A.

Binding site surface, hydrogen bonds and van der Waals radius were calculated with their respective tools in the software and performed using its default setting, and each measured parameter was stored in its respective file. We selected in the display menu, show only the H-bonds for each individual residue.

### 2.3 | Plasmid and site direct mutagenesis

GST-PLCδ1PH domain wild-type sequence cloned in plasmid pGST3 and mutant GST-PH domain sequence R40A (pGST3-R40A) were a gift from Dr Hitoshi Yagisawa.[23]

Wild type PH-domain DNA sequence was mutated using whole plasmid site direct mutagenesis.[24] The following primers were designed to perform the mutations: for K30A: fw 5′-CCAGCTTCTGC GTGTGAAGTCCA-3′; rv 5′- TGGACTTCACACGCAGAAGCTGG-3′. For W36F: fw 5′-GTCCAGCTCGTTTCGTAGGGAAC-3′; rv 5′-GTTC CCTACGAAACGAGCTGGAC-3′. For W36Y: fw 5′GTCCAGCTCGT

ACCGTAGGGAAC-3′; rv 5′GTTCCCTACGGTACGAGCTGGAC-3′. We used a PCR with 30 rounds, denaturalization temperature was 94°C, anneal temperature to 60°C, and the extension temperature was 72°C. Platinum Pfx polymerase (thermofisher) was used. To degrade WT sequence, we used DPN1 enzyme (New England Biolabs) for 1 hour directly in the PCR tubes. The mutant plasmids were named pGST3-R30A, pGST3-W36F, and pGST3-W36Y. We used 5 µl to transform bacterial cells.

## 2.4 | Recombinant peptides expression and purification

All of the recombinant peptides were expressed in *E. coli* (DE3)-pLysS (Stratagene, La Jolla, CA, USA). The transformation of both strains was performed by the heat shock method. Expression was induced with 1-isopropyl-β-D-thiogalactopyranoside (IPTG) 0.1 M in LB medium for 2 hours at 37°C and 200 rpm. Bacterial cells were centrifuged at 3000 rcf and 4°C. The pellet was resuspended and sonicated. After this, we centrifuged at 10 000 rcf and 4°C. Soluble fraction was recovered for its further purification.

Purification of the peptides was achieved using a column with glutathione-sepharose 4B resin, subsequently; domains were recovered from said matrix under mild elution with glutatión10 mM to preserve the antigenicity and functionality of the peptides. Purified elution's from each mutant were normalized and loaded in a 12% SDS-PAGE and stained with coomassie stained.

## 2.5 | Western blot assay

We used 50 ng of the normalized domains and check for western blot comparison. Each elution was loaded in a 12% SDS-PAGE. Subsequently, the proteins were transfer to a nitrocellulose membrane that was blocked with PBS-Tween 20 (1%) and 3% of BSA at room temperature. Later was made an incubation with anti-PH domain rabbit antibody (1/5000) for 2 hours at room temperature and a third with secondary antibody (1/4000) 1 hour at room temperature, with 3 washes between incubations and revealed with chemoluminescence (Amersham ECL Prime Western Blotting Detection Reagent).

## 2.6 | Fatblot assays

To test the affinity of each domain to different PIPs, the purified domains were tested on PIP Strips (Echelon Biosciences Inc, California) with 100 pM of different PIPs, including the 7 PIPs. Strips were blocked with PBS-Tween 20 (1%) and 3% of BSA or 1 hour at room temperature, then three 10-minute washes with PBS-T were made. After, strips were incubated first with 0.5 µg/ml of each domain over night at 4°C. Subsequently, we used the same protocol for incubations with the primary and secondary antibodies in the western blot assay.

# 3 | RESULTS

Based on the amino acid sequence of the PLCδ1 PH domain from *Rattus norvegicus* (the domain nucleotide sequence has 426 nucleotide bases), NCBI database yielded a total of 100 similar sequences to the PLCδ1 PH domain. Nine of them were selected with a lower percentage of similarity, but a confirmed affinity for PIPs (see Section 2). We analyzed the amino acids directly involved in the binding of PLCδ1 PH domain to PIPs (PMID 8521504). These amino acids are lysines 30 (K30) and 32 (K32), tryptophan 36 (W36), arginine in position 40 (R40), glutamate 54 (E54), and serine 55 (S55) (Figure 1A). Two glycines are conserved in all sequences. Figure 1 shows the number of amino acids that are conserved in more than 60% of the sequences analyzed. Generally, these are amino acids with positive charges—histidine 57 and arginine 76 (with a conservation percentage of 90%) and aliphatic amino acids such as isoleucine 99 with 90% of conservation. Phenylalanine 97 and alanine 112 has a conservation percentage of

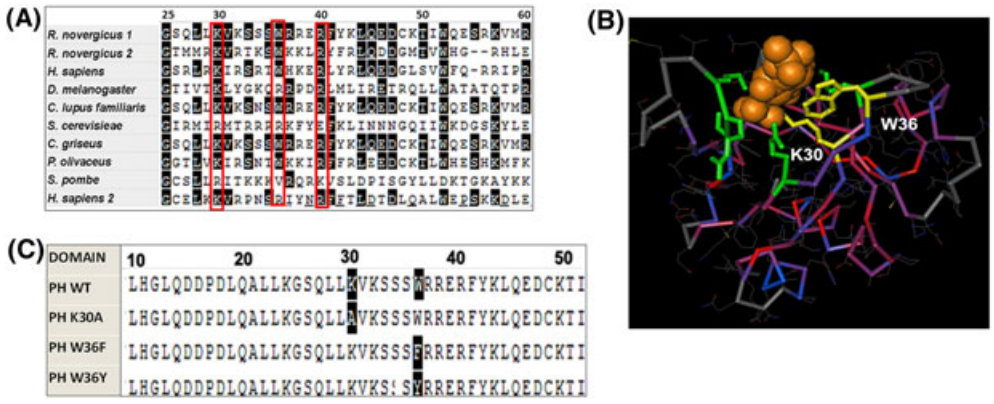


**FIGURE 1** Selection of residues for site direct mutagenesis. A, Sequence alignment of distinct PH domains made by the method MUSCLE. Sites conserved by 50% or more are in black. Red boxes indicate amino acids selected for mutagenesis. B, Representation of the tertiary structure of PH domain. Residues highlighted in green are the amino acids essential for the recognition of PI(4,5)P2 by the PH domain. Yellow amino acids have been selected for the mutagenesis in this study. The color scale from gray to red shows the percentage of conservation for each amino acid residue, with the gray showing less conserved amino acids and the red showing those preserved in 100% of the sequences. The red ones have been used to compare a sequence alignment. Orange molecule IP3 is in orange and has been used to determine the interaction of phosphate groups. C, Sequence alignment with the residues 9 to 51 of the wild type (WT) and mutant (K30A, W36F, and W36Y) PH domains. Nucleotide sequences were obtained after sequencing to confirm amino acid changes, aligned and translated into their respective amino acid sequences in the program MUSCLE. Residues in black are those changed relative to the wild type PH domain

70%, while tryptophans 52 and 121 are conserved in 80% of the sequences (Figure 1A). One can observe a match between some of the amino acids conserved in the alignment and those involved in the recognition of phosphoinositides. These same amino acids are conserved in 50 or higher percentage except for tryptophan 36 and serine 55 (Figure 1B). We replaced lysine 30 by alanine and tryptophan 36 by phenylalanine or tyrosine (Figure 1C).

Purified recombinant domains were analyzed by PAGE. Only bands corresponding to PH domain, both wild type and mutants, with a molecular weight of 46 kDa were observed in each gel (Figure 2A, lines 1-5), confirming the purification of the domains. To determine the functionality of the antibodies against the PH domain, we used Western blotting and revealed the bands corresponding to the PH domains of the appropriate size (Figure 2B). Fat blots were performed to check whether the mutant PH domains differed in the specificity compared to the wild type domain (Figures 2C, lines 1-5). Wild-type domain binds strongly to $PI(4,5)P_2$ and more weakly to PI(5)P, $PI(3,4)P_2$, and $PI(3,4,5)P_3$ (Figure 2C, line 1). Pleckstrin homology domain with a mutation R40A did not recognize any phosphoinositide (Figure 2C, line 2). In the case of K30A mutant, the fat blot showed that it was capable of binding with a similar specificity to $PI(4,5)P_2$, $PI(3,4,5)P_3$, and PA (Figure 2C, line 3). W36Y mutant appeared to interact with the PA, PI(4)P, $PI(3,4)P_2$, and $PI(3,5)P_2$, while its binding to PI(5)P, $PI(4,5)P_2$, and $PI(3,4,5)P_3$ is much stronger (Figure 2C, line 4). W36F mutant bound all phosphoinositides, although the signal was less intense for PIPs monophosphates as compared to PIPs bisphosphates and PIP triphosphate. It also interacted with PA (Figure 2C, line 5).

To explain the different PIP specificities showed in fat blots, we measured 3 parameters for each domain. The first was the surfaces and cavities in the binding site of the domains. Surface of the wild type PH domain (Figure 3A) near to the fifth $PO_4^{3-}$ of $IP_3$ differs from the respective area of the R40A mutant (Figure 3B), which seemed to be reduced in this position. A minimal difference appeared to occur in the total surface of the mutant K30A (Figure 3C); however, we observed a slight reduction of the surface very close to the fourth and fifth $PO_4^{3-}$. W36Y and W36F mutants showed significant changes in their surfaces (Figure 3D,E). These changes appeared in the center of the binding site and near the first $PO_4^{3-}$. Van der Waals radius was calculated to see steric impediments and charge differences. Compared with wild type domain (Figure 3F), a loss of a positive charge in the residue 40 in the R40A mutant (Figure 3G) and a predominant presence of a negative charge from E54 near to a negative charge of the fifth $PO_4^{3-}$ of $PI(3,4,5)P_3$ were observed. A loss of a positive charge in the mutant K30A and a predominance of negative charges were observed; however, it seemed to cause less drastic changes in a charge, a size, and a form of the binding site as compared to the wild type domain (Figure 3H). Mutant W36Y showed a bigger binding site (Figure 3I) and an additional negative charge, corresponding to the hydroxyl group of tyrosine, unlike to mutant W36F, which was otherwise similar to the mutant W36Y (Figures 3J).

Hydrogen bonds (H-bonds) were calculated for each wild type and mutant domains. Wild type R40 could form 2 H-bonds with the fifth $PO_4^{3-}$ of the substrate $IP_3$ (Figure 3K). Mutant residue A40 was not able to form any H-bond with substrate (Figure 3L). Residue K30 formed 2 H-bonds with the forth $PO_4^{3-}$ and 1 H-bond with the fifth $PO_4^{3-}$ in the substrate as well as 2 additional H-bonds with E54. Mutant A30 formed only H-bonds with E54 (Figure 3M). Wild type W36 formed 1 H-bond with the fourth $PO_4^{3-}$ in the substrate and 1 H-bond with K32. Both mutant residues Y36 (Figure 3N) and F36 (Figure 3O) maintained only their H-bonds with K32.

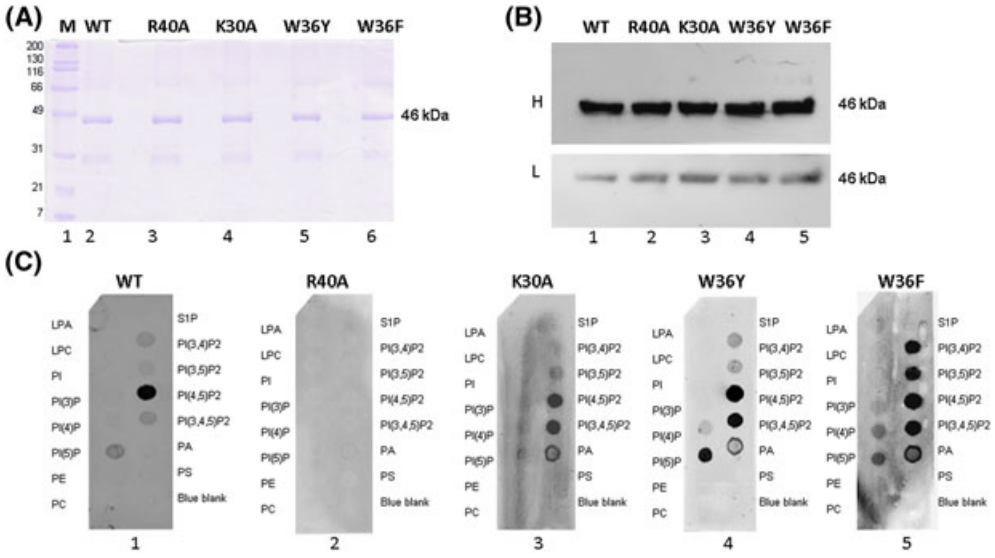


**FIGURE 2** Purification of the wild type and mutated PH domains and determination of their specificities. A, Electrophoresis in 10% polyacrylamide gel (SDS-PAGE) of the purified wild type PH domain (line 1) and the mutated PH domains: R40A (line 2), K30A (line 3), W36Y (line 4), and W36F (line 5). B, Immunodetection of the wild type (line 1) and mutant (lines 2–5) PH domains as stated on the figure. H and L represent a high and low exposure in the film for quantitative comparison. C, determination of the specificities of the domains by fat blot assay. Line 1, fat blot of wild type PH domain. It recognized PI(5)P, PI(4,5)P2, PI(3,4)P2, and PI(3,4,5)P3. Line 2, fat blot of the mutated PH R40A domain. It did not recognize any of the PIPs present in the PIP strip. Line 3, fat blot of the mutated PH K30A domain. It bound with a similar specificity to PI(4,5)P2, PI (3,4,5)P3, and PA. Line 4, fat blot of the mutated PH W36Y domain. It interacted with the PA, PI(4)P, PI(3,4)P2, and PI(3,5)P2, while its binding to PI(5)P, PI(4,5)P2, and PI(3,4,5)P3 is much stronger. Line 5, fat blot of the mutated PH W36F domain. The domain interacted with all phosphoinositides; the signal was less intense for PIPs monophosphates as compared to PIPs bisphosphates and PIP triphosphate; it also interacted with PA

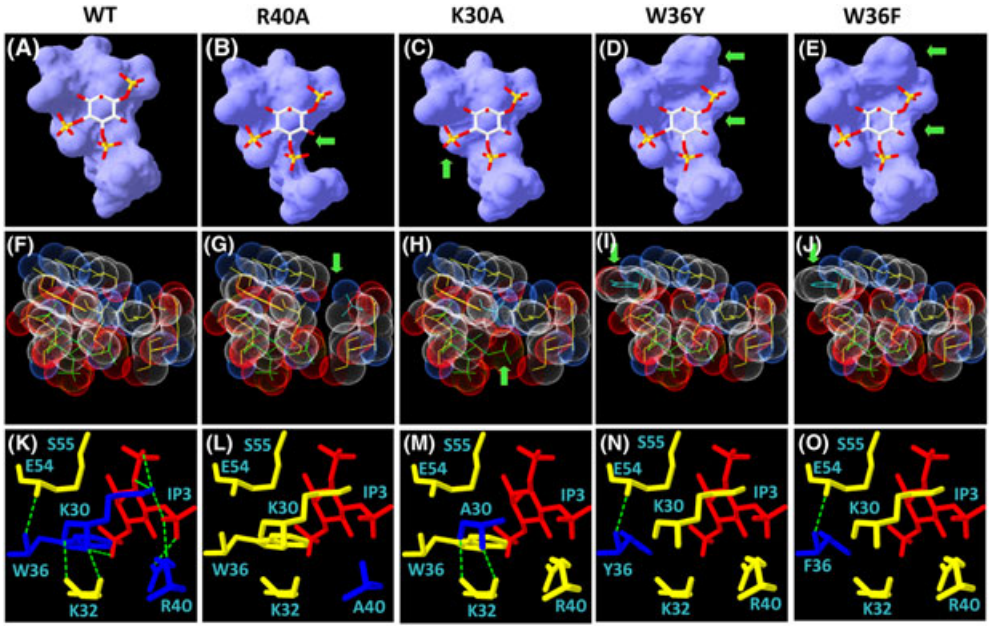


**FIGURE 3** Surface changes in PIP recognition site of wt and mutants PH domains. After in silico mutations performed in Swiss-PDBV software. Calculation of molecular surface of the binding site of each domain was performed of both surfaces is shown. A, wt molecular surface; B, R40A PH domains surface; C, K30A PH surface; D, W36Y PH surface E, W36F surface. Van der Waals radius of each residue in wild type and mutant domains. F to J, a first view of van der Waals radius of residues. The residues in the binding site are showed in yellow. Mutagenic residues are in blue. IP3 molecule is highlighted in green. Positive charges are highlighted in strong blue; the negatives are in red, and neutrals are in white. K to O, H-bonds presents in the bindig pocket of wt and mutant domains. Residues in the binding site are showed in yellow, except the selected residue for mutagenesis that are showed in blue. H-bonds are showed in green doted lines. IP3 molecule is colored with red. K, wt Hbonds; L, R40A H-bonds; M, K30A H-bonds; N, W36Y H-bonds; O, W36F H-bonds. Main differences were highlighted with red arrows

## 4 | DISCUSSION

The alignment performed for this and previous work[15,17,18,23,25] suggests that amino acids K30, W36, and R40 are indispensable for the binding of PIPs at the PH domain of PLCδ1. These studies have also studied alanine effect but have been confined mostly to the evaluation of the domain affinity to $PI(4,5)P_2$ without any attempt to explain the mechanisms, which control these changes, otherwise attributed only to the charge effects.[17,18,26-30] To explore the mechanisms in details and fill up the gaps, the mutations of a basic (K30) and an aromatic (W36) residues were conducted in this study.

The substitution of lysine 30 in the PLCδ1 PH domain resulted in the specificity of the mutant K30A to $PI(4,5)P_2$, $PI(3,4,5)P_3$, and PA. The residue K30 appears to be necessary for recognition of the $PO_4^{3-}$ group at carbon 3 of the inositol ring, which would explain the apparent changes in the K30A mutant specificity.[17] Unlike lysine, alanine has no charge and is a nonpolar amino acid.[31] As mentioned, the charge plays a fundamental role in the domains binding of PIPs; because of this, we expected a total disruption with any PIP interaction, and however, only the specificity of the mutant domain was observed.

Additionally, the molecular weight of these amino acids is very different: alanine has 89.09 Da, while lysine has 146.19 Da. It seems that because of both factors, a charge and a molecular weight, the mutant K30A requires a higher negative charge to recognize phosphoinositides, preferring those phosphorylated at the third position of the inositol ring. Unlike the mutant R40A, which loses the H-bonds with the substrate, the mutant K30A maintains its capability to bind PIPs; however, it seems that the residue W36 could be enough to hold the fourth $PO_4^{3-}$ of the substrate at the binding site.

It is known[7] that the PH domain recognition sites for the phosphoinositides are composed of the residues with positive charges, which are responsible for the binding of the negative charges of the phosphoinositides $PO_4^{3-}$ groups. Besides, in the majority of the analyzed domains, there is at least 1 aromatic residue like tryptophan. The W36F mutant was able to bind to the 7 species of phosphoinositides more intensively than the W36Y mutant. Tryptophan 36 is involved in the recognition of the $PO_4^{3-}$ at the fifth position of the inositol ring of $PI(4,5)P_2$ in conjunction with arginine 40.[17,18] Tryptophan is the amino acid of higher molecular weight (204.23 Da) and is considered to replace a smaller but chemically similar amino acid like phenylalanine, which may cause the increase of the binding pocket and a decrease in the discrimination of the PIPs. This could cause steric interference, which contributes to a correct orientation of the $PO_4^{3-}$ groups of $PI(4,5)P_2$ at the binding site of the wild type PH domain and finally favors to the binding of $PI(4,5)P_2$ against other phosphoinositides.[32] Perhaps, because of this effect, the W36F mutant seems to be able to bind to a greater number of PIPs.

Phenylalanine has a molecular mass of 165.19 Da, but also it is more hydrophobic than tryptophan,[31] which could explain that this mutant may also bind the PA as a hydrophobic molecule. Mutant W36Y binds weaker the PA and recognized all phosphoinositides except PI(3)P. The binding of W36Y to PI(5)P, $PI(4,5)P_2$, and $PI(3,4,5)P_3$ appears to be more intense. The difference in the specificity between the mutants W36Y and W36F may be due to the hydroxyl group of tyrosine, which makes this amino acid polar and more reactive

than phenylalanine. Moreover, the negative charge of the hydroxyl group of tyrosine could repulse the fourth $PO_4^{3-}$.The observed differences in the specificity may also be due to the changes in the tertiary structure of the domain or the domain conformational changes, which allow the interactions with a larger number of PIPs or contribute to the greater binding forces. In support of this, some authors have suggested the importance of allosteric interactions, which cause the changes in the affinity of the PH domain.[25,30,33]

## 5 | CONCLUSIONS

We showed evidence that support the idea that aromatic amino acids plays a pivotal role in the specificity of the PH domain, forming binding pockets with different sizes and shapes, contributing with the electrostatic interaction, discriminating certain PIPs, and favoring interaction with others. The mutant domain K30A recognized PI(4,5)$P_2$, PI(3,5)$P_2$, and weakly PI(3,4,5)$P_3$, whereas the mutant PH domain W36F was bound to the 7 phosphoinositides, as well as phosphatidic acid. The mutant PH domain W36Y appears to bind phosphatidic acid and to all phosphoinositides with the exception of PI(3)P. The binding is more intense in case of PI(5)P, PI(4,5)$P_2$, and PI(3,4,5)$P_3$. These observations go against the notion that a mutation in positively charged amino acids would cause a disruption in the interaction between PH domain and PIPs. This would help to design better lipid biosensors that allow for more accurate results and easier to interpret. However, it is necessary to investigate the role that posttranslational modifications could play, eg, phosphorilations, in the regulation of PIP-binding proteins in their specificity, since cells must have mechanisms that make theirs proteins specific to a particular PIP, increasing its affinity for it.

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