Phosphotyrosine protein of molecular mass 30 kDa binds specifically to the positively charged region of the pleckstrin N-terminal pleckstrin homology domain

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It has been proposed that phosphoinositides and inositol phosphates serve as general ligands for members of the structurally related pleckstrin homology (PH) domain family. The N-terminal PH domain of pleckstrin (N-PH), in contrast with other PH domains, does not bind to any of these ligands with the high affinity expected for a physiological interaction. To examine whether N-PH might instead mediate protein–protein interaction, a fusion protein with glutathione S-transferase (GST) expressing N-PH (GST–N-PH) was used to screen [³⁵S]methionine metabolically labelled HL-60 and Bac1.2F5 cell lysates for potential binding partners. A 30 kDa binding protein was identified in both cell lines. Binding to N-PH demonstrated specificity, because binding was approx. 10-fold higher than when an equimolar amount of pleckstrin C-terminal PH domain (GST–C-PH) was used as probe. The 30 kDa protein could also

be metabolically labelled with [³²P]P_i and proved to be a tyrosine-phosphorylated protein. Binding to N-PH could be specifically inhibited with phosphotyrosine but not with phosphothreonine; the inhibition was concentration-dependent. Site-directed mutagenesis indicated that a positively charged region previously identified as the phosphoinositide-binding site in N-PH and other PH domains, rather than a putative phosphotyrosine-binding region previously identified in structurally similar phosphotyrosine-binding (PTB) domains, served as the binding site. These results suggest that the positively charged region of N-PH has the potential to interact with a protein ligand that contains phosphotyrosine.

Key words: phosphotyrosine-binding domain, protein kinase C substrate, signal transduction.

INTRODUCTION

The pleckstrin homology (PH) domain is a structurally conserved ligand-binding motif found in more than 100 proteins and thought to be involved in intracellular signalling [1–4]. It is composed of approx. 100 amino acids. The amino acid sequence identity between different PH domains is generally low; in contrast, the three-dimensional folded structure of these domains is highly conserved (structural information is available for seven PH domains; presented in [5]). The complete domain is folded into a β -barrel structure that contains two β -sheets. On the first β -sheet there is a positively charged region formed by several basic residues. Structural studies have shown that this positive region can serve as the binding site for phosphoinositides. The interaction between basic amino acid side chains and the phosphates of the inositol ring accounts for the major forces for binding [6–10]. It is thought that this binding could serve either to recruit the protein to the plasma membrane or to regulate protein function allosterically. Although a similar positive region has been found in all known PH domains, many of the PH domains do not bind to phosphoinositides with the high affinity that would be expected for a physiological interaction [5,6,11,12] (reviewed in [13–15]). It is reasonable to expect that such a diverse group of proteins with only limited sequence identity should bind to a wider range of ligands than those currently reported. Therefore for many of these domains the identity of their physiological ligands remains unclear.

Some PH domains have already been shown to interact with protein ligands; this binding might also involve regions other than the positively charged surface, which can bind phosphoinositides. For example, the $\beta\gamma$ subunits of G-proteins (G _{$\beta\gamma$}) can bind to many PH domains [16–19]. Binding involves the C-terminal α -helix of the PH domain and requires an additional short stretch of amino acids beyond the domain for high-affinity binding. For the β -adrenergic receptor kinase (β ARK), the binding of G _{$\beta\gamma$} and phosphoinositides seems to be synergistic and together they enhance membrane translocation of the kinase [20]. It has also been reported that protein kinase C (PKC) isoforms can bind to some PH domains at a site that competes with PtdIns(4,5)P₂ for binding [21,22] and that the insulin receptor substrate 1 (IRS-1) PH domain can bind to acidic motifs in proteins through an unspecified site [23]. In addition, the strong structural similarity between PH domains and phosphotyrosine (p-Tyr)-binding (PTB) domains [24,25] suggests that some PH domains might have the potential to mediate protein–protein interaction through a conserved L-shaped cleft responsible for peptide ligand binding to PTB domains [26–29], although no such ligands have yet been reported for PH domains. PTB domains have already been shown to bind, in some cases simultaneously, to two different ligands, through either independent or possibly overlapping sites [27,30–32]. Therefore, because only the structures of the PH domains, not their primary amino acid sequences, are conserved, and because of the potential for more than one binding region and for interaction between

Abbreviations used: CSF-1, colony-stimulating factor 1; C-PH, C-terminal PH domain of pleckstrin; DEP domain, the sequence between the PH domains, consisting of D [*dishevelled* gene product (dsh)] plus E (egl-10) plus P (pleckstrin); GST, glutathione S-transferase; IRS-1, insulin receptor substrate 1; N-PH, N-terminal PH domain of pleckstrin; PH domain, pleckstrin homology domain; PKC, protein kinase C; PTB domain, phosphotyrosine-binding domain; p-Tyr, phosphotyrosine.

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multiple binding regions, it has proved difficult to identify physiological ligands for many PH domains.

Pleckstrin is a 40 kDa protein found exclusively in haemopoietic cells. It is rapidly phosphorylated in many of these cells after activation of PKC and thus is thought to be a signalling protein in the PKC pathway [33]. Pleckstrin contains two PH domains, which are located at its N-terminus and its C-terminus respectively [34]. The N-terminal PH domain (N-PH) is followed immediately by the PKC phosphorylation site [35,36]. The PH domains are separated by a DEP domain, named for D [*dishevelled* gene product (*dsh*)] plus E (*egl-10*) plus P (pleckstrin); this is a conserved motif found in proteins that are thought to regulate GTP/GDP exchange but whose function otherwise remains unclear [37]. Neither of the two PH domains of pleckstrin binds to phosphoinositides [6] or to any known protein ligand with the high affinity expected for a physiological interaction. It is possible that (1) phosphorylation by PKC could affect binding, (2) oligomerization of the pleckstrin protein could enhance binding (as has been shown for dynamin [38]), or (3) the simultaneous binding of two ligands could synergistically enhance binding (as shown for the β -adrenergic receptor kinase [20]), but this remains to be shown for pleckstrin. The present study was designed to identify protein ligands that might interact with N-PH and to characterize the interaction. Because of the likelihood that post-translational modification, specifically phosphorylation, might be required for efficient binding, expression screening in *Escherichia coli* and two-hybrid screening were not used in this study. We chose instead to prepare a recombinant fusion protein, GST-N-PH, of glutathione S-transferase (GST) with N-PH to screen haemopoietic cell lysates for potential binding partners and to map the region of N-PH responsible for binding.

EXPERIMENTAL

Materials

The full-length pleckstrin cDNA was amplified from HL-60 cell RNA by reverse-transcriptase-mediated PCR. DNA sequencing showed it to be the same as the previously reported sequence [34]. The GST fusion protein expression vector pGEX-4T-1 and the glutathione-Sepharose beads used to purify the expressed fusion proteins were obtained from Pharmacia (Piscataway, NJ, U.S.A.). All fusion proteins were expressed in the BL21 strain of *E. coli* and purified in accordance with the manufacturer's protocol. Oligonucleotide primers for mutagenesis were synthesized by Gibco (Grand Island, NY, U.S.A.). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, U.S.A.). *Pfu* DNA polymerase was obtained from Stratagene (La Jolla, CA, U.S.A.). Plasmid isolations were performed with QIAprep spin columns from Qiagen (Chatsworth, CA, U.S.A.). Tran³⁵S-label ([³⁵S]methionine/[³⁵S]cysteine, 40–45 TBq/mmol) and [³²P]P_i (320 TBq/mmol) were obtained from ICN (Costa Mesa, CA, U.S.A.) and NEN (Boston, MA, U.S.A.) respectively. Anti-(p-Tyr) monoclonal antibody PY-20 was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Enhanced chemiluminescence (ECL[®]) kit was from Amersham (Little Chalfont, Bucks., U.K.) and Kodak X-ray film was used for immunoblot analysis. Protein concentrations were determined by using Dye Reagent from Bio-Rad (Richmond, CA, U.S.A.). The HL-60 (human promyelocytic leukaemia) cell line was from A.T.C.C. (Manassas, VA, U.S.A.). The Bac1.2F5 cell line was obtained from Dr. E. R. Stanley (Albert Einstein College of Medicine, Bronx, NY, U.S.A.). Dulbecco's modified Eagle's medium and RPMI 1640 cell culture medium were produced by Gibco.

Cell culture

HL-60 cells were grown in suspension culture in RPMI 1640 medium with 10% (v/v) fetal calf serum. Bac1.2F5 cells were grown in Dulbecco's modified Eagle's medium with 10% (v/v) newborn calf serum. Bac1.2F5 cells are differentiated mouse macrophage-like cells that are dependent on colony-stimulating factor 1 (CSF-1) for growth [39]. The CSF-1 was provided by the condition medium from a mouse L-cell culture (A.T.C.C.). CSF-1-treated Bac1.2F5 cells are adherent and retain their differentiated phenotype as they divide in culture. All cells used in this study were grown at 37 °C in a humidified incubator under air/CO₂ (93:7).

Metabolic labelling experiments

[³⁵S]Methionine labelling of HL-60 cells

An exponentially growing suspension culture (5 × 10⁵ cells/ml) was spun down and re-fed with fresh medium 18 h before labelling. For labelling, 1 ml of the culture was pelleted by centrifugation and washed twice with PBS, pH 7.4. The cells were resuspended with 1 ml of methionine-free RPMI 1640 with 10% (v/v) dialysed newborn calf serum. After incubation for 1 h, Tran³⁵S-label ([³⁵S]methionine/[³⁵S]cysteine) was added at 100 µCi/ml of medium. The labelling continued for 4 h with periodic agitation. After labelling, cell lysate from 5 × 10⁵ cells was used in each protein binding assay, as described below.

[³⁵S]Methionine labelling of Bac1.2F5 cells

One 60 mm plate of Bac1.2F5 cells at 70% confluence was re-fed with fresh medium 18 h before labelling. For labelling, cells were washed twice with PBS and incubated in 3 ml of methionine-free medium for 1 h. Tran³⁵S-label was added at 100 µCi/ml of medium and the cells were further incubated for 4 h before harvesting. After labelling, cell lysate from one plate was used in each protein binding assay, as described below.

[³²P]P_i labelling of Bac1.2F5 cells

One 60 mm plate of Bac1.2F5 cells at 70% confluence was fed with fresh medium 18 h before labelling. For labelling, cells were washed twice with Tris-buffered saline, pH 7.4, then incubated with 3 ml of phosphate-free medium for 30 min. [³²P]P_i was added at 1 mCi/ml of medium, after which the cells were incubated for 1.5 h. After labelling, cell lysate from one plate was used for each protein binding assay, as described below.

Protein binding assay

Unlabelled Bac1.2F5 cells were grown to approx. 80% confluence in 100 mm dishes for use in the binding assay. Cells were washed twice with ice-cold PBS, collected in an Eppendorf tube and then lysed in 1 ml per plate of lysis buffer [50 mM Tris/HCl (pH 7.5)/120 mM NaCl/0.5% (v/v) Triton X-100/90 mM NaF/1 mM sodium pyrophosphate/200 µM sodium orthovanadate/10 µg/ml aprotinin/10 µg/ml leupeptin/10 µg/ml soybean trypsin inhibitor/1 mM dithiothreitol/5 mM EDTA/5 mM EGTA] by incubation on ice for 30 min. The Triton-soluble lysate supernatants were collected after a 5 min microcentrifuge spin and pooled for use in the binding assay. The Triton-soluble lysates were precleared three times with GST protein bound to glutathione-Sepharose beads to eliminate non-specific binding. The total amount of GST protein used in the preclearing steps was approx. 3-fold greater than that of GST-N-PH (or other

Figure 1 Schematic diagram of fusion protein constructs prepared with the pleckstrin cDNA

Three GST fusion protein constructs were designed by using the pleckstrin cDNA: (1) GST-N-PH, containing only the N-terminal PH domain (amino acid residues 1–105), (2) GST-DEP, containing the sequence between the PH domains (residues 103–240), and (3) GST-C-PH, containing the C-terminal PH domain (residues 230–350). In each case the GST sequence was located at the N-terminus of the expressed fusion protein. The small black rectangle (residues 107–120) shows the location of the PKC phosphorylation sites. A fourth construct was prepared to express only the GST protein itself for use as control. Primers and procedures used to prepare the constructs are described in the Experimental section.

fusion protein) ultimately used in the binding assay. When cells were collected from more than one plate, cell lysates were pooled, precleared and then aliquoted into the binding assay. For each binding assay, 500 μ l of precleared lysate was mixed with a standard amount of the indicated GST fusion protein to give a final concentration of binding protein of approx. 3.5 μ M. In all cases, the GST fusion proteins were prepared within 48 h of their use in the binding assay. The protein concentrations of the unlabelled cell lysates used in these assays ranged from 5.0 to 8.0 mg/ml. A titration of the binding assay with the 30 kDa protein (identified in the Results section) and GST-N-PH showed that in this range the strength of the binding signal intensity increased with the protein concentration of the lysate (results not shown). Binding was performed on ice for 1 h. The beads were rapidly spun down and washed three times with lysis buffer to remove unbound protein. The binding samples were then subjected to SDS/PAGE and transferred to nitrocellulose. The protein bands were detected by Western blot analysis or by autoradiography or PhosphorImager scan for metabolically labelled proteins.

Immunoblotting with PY-20 anti-(p-Tyr) antibody

The proteins in the SDS/polyacrylamide gel were transferred to nitrocellulose with a Western blot transfer protocol. The nitrocellulose was blocked in TSB [10 mM Tris/HCl (pH 7.5)/154 mM NaCl/5% (w/v) BSA] overnight at 4 °C, then incubated with 1:1000 diluted PY-20 antibody for 1 h at room temperature. After a thorough washing with 10 mM Tris/HCl (pH 7.4)/0.9% NaCl/0.1% (v/v) Triton X-100, the nitrocellulose was incubated with a 1:10000 dilution of peroxidase-linked anti-mouse IgG antibody (Amersham) for 30 min at room temperature. The nitrocellulose was then washed again three times for a total of 30 min. The signals were detected by using the enhanced chemiluminescence kit in accordance with the manufacturer's protocol.

Generation of truncated and mutant constructs

Three segments of the pleckstrin cDNA were used to prepare GST fusion protein constructs for expression in *E. coli*. The constructs, referred to as GST-N-PH, GST-DEP and GST-C-PH, encoded the N-terminal PH domain, the DEP domain and the C-terminal PH domain of pleckstrin respectively (Figure 1). Seven mutant N-PH fusion protein constructs were also pre-

pared: N-PH-K51A, N-PH-K75A, N-PH-KK51/75AA, N-PH-K13A, N-PH-K14A, N-PH-K22A and N-PH-K45A, in which the lysine residue or residues of interest were converted to alanine. All truncated and mutant pleckstrin expression constructs except the GST-C-PH construct were generated by PCR with *Pfu* DNA polymerase [40]. The original full-length pleckstrin was used as the template for all PCRs, except for the generation of N-PH-KK51/75AA, which used N-PH-K51A as the template. PCR conditions were 36 cycles of 15 s at 94 °C for denaturation, 30 s at 55 °C for annealing, and 2 min at 72 °C for extension. The final PCR products were digested with *Bam*HI and *Xho*I for in-frame ligation in pGEX-4T-1 vector. The C-PH fragment (residues 230–350) was excised directly from full-length pleckstrin by using *Eco*RI and *Xho*I to produce the GST-C-PH construct. The primers used in PCR were as follows, in which the nucleotides altered to introduce restriction sites (NPH-F, NPH-R, DEP-F, DEP-R) or to introduce mutations (all other primers) are underlined: DEP-F, 5'-CATTGGATCCATTGAAGGAGGCCA-3'; DEP-R, 5'-CCCCCTCGAGTTCCTTCTTTGAGAT-3'; GST(-210)-F, 5'-CCTGACTTCATGTTGTATGACGCTC-3'; NPH-F, 5'-GCGTGGATCCAGCATGGAACCAAA-GCGG-3'; NPH-R, 5'-ATTTCTCGAGTCCTTCAATGCATTT-3'; NPH-K13A-F, 5'-CTTGTGGCGAAGGGGAGCGGTGTTCAATACG-3'; NPH-K13A-R, 5'-GCTCCCCCTTCGCCACAAAGTAGCCCTCTCT-3'; NPH-K14A-F, 5'-CTTGTGAA-GGCGGGGAGCGTGTTCATACG-3'; NPH-K14A-R, 5'-GCTCCCCGCTTCACAAGGTAGCCCTCTCT-3'; NPH-K22A-F, 5'-CGTGGGCACCCATGTGGGTTGTATTG-3'; NPH-K22A-R, 5'-CCACATGGGTGCCACGATTGAACACGC-3'; NPH-K45A-F, 5'-AGCCCCGACAGGAATGATCCGCTGAAAGG-3'; NPH-K45A-R, 5'-CATTCCTGCGGGGCTGTTGTCACTTTTC-3'; NPH-K51A-F, 5'-CCGCTG-GCAGGGAGCACTCTGACTAGC-3'; NPH-K51A-R, 5'-GCTCCCTGCCAGCGGGATCATCTTTG-3'; NPH-K75A-F, 5'-ACGACCGCACAGCAGGACCACTTCTTC-3'; NPH-K75A-R, 5'-CCTGCTGTGCGGTCGTAGTGATCTTAAAC-3'.

The sequences of all constructs were confirmed by DNA sequencing (DNA Sequencing Facility, SUNY Brooklyn, NY, U.S.A.). Yields of the mutant GST fusion proteins expressed in *E. coli* were similar to that of the GST-N-PH wild-type protein. Binding assays were performed with the same concentration of wild-type or mutant protein in each case.

RESULTS

N-PH binds to a 30 kDa protein

Using the purified GST-N-PH fusion protein to screen a ³⁵S-labelled HL-60 cell lysate, we found consistently that a protein with an apparent molecular mass of 30 kDa on SDS/PAGE bound to N-PH (Figure 2A, lane 1). This protein was not observed when the binding was performed with either the GST-DEP fusion protein or when precleared with the GST protein alone (Figure 2A, lanes 2 and 3). With the use of the same approach to screen a metabolically labelled Bac1.2F5 cell lysate, which is a more differentiated macrophage-like cell line, the 30 kDa protein consistently gave the strongest signal (Figure 2B) under the same conditions.

One other metabolically labelled protein was consistently observed when N-PH was used as probe: it migrated with a molecular mass of approx. 43 kDa (Figures 2A and 2B). Another protein with a molecular mass of approx. 80 kDa is visible in Figure 2(A) but was not observed consistently during our experiments. Possible identities of these labelled proteins will be addressed in the Discussion section.

Figure 2 Binding of a 30 kDa protein to N-PH

HL-60 cells (**A**) and Bac1.2F5 cells (**B**) were metabolically labelled with [^{35}S]methionine. Labelled cells were lysed, precleared for non-specific binding with control GST protein (GST lanes) and the supernatants were used in binding assays with the indicated GST fusion proteins, as described in the Experimental section. The bound protein was subjected to SDS/PAGE, transferred to nitrocellulose and detected by autoradiography. In (**A**), binding assays with HL-60 cell lysates showed that a 30 kDa protein (arrowed) bound to N-PH. As shown in (**B**), the 30 kDa protein (arrowed) was also observed when the binding assay was performed with labelled Bac1.2F5 cell lysate instead. The signal in (**B**) was detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) by using ImageQuant software. The positions of molecular mass markers are indicated at the left and at the right.

Identification of the 30 kDa binding protein as a p-Tyr protein

To examine whether the bound 30 kDa protein was a phosphorylated protein, the binding assay was performed instead

Figure 3 Bound 30 kDa protein is a p-Tyr protein

Left panel: [^{32}P]P_i was used to label Bac1.2F5 cells metabolically. Labelled cell lysate was incubated in the binding assay with GST-N-PH, as described in the legend to Figure 2, and bound protein was detected by autoradiography. A 30 kDa ^{32}P -labelled phosphoprotein was observed as indicated, and its migration coincided with that of the ^{35}S -labelled 30 kDa protein shown in Figure 2. Right panel: the binding assay with GST-N-PH was repeated with unlabelled Bac1.2F5 cell lysate. The bound protein was subjected to Western blot analysis and detected with the anti-(p-Tyr) antibody PY-20. The strong signal observed at 30 kDa is indicated. The migration of the unlabelled GST-N-PH fusion protein, which might give a non-specific signal in these assays due to its abundance, is also indicated in both panels. The positions of molecular mass markers are indicated at the left.

Figure 4 Free p-Tyr inhibits the binding of the 30 kDa protein to N-PH

(**A**) The binding assays with GST-N-PH and unlabelled Bac1.2F5 cell lysate were repeated in the presence of increasing concentrations of free p-Tyr. Migration of the 30 kDa protein was detected by Western blot analysis with the anti-(p-Tyr) antibody (top panel). The addition of p-Tyr produced a concentration-dependent inhibition when the strength of the signal intensity was analysed with the NIH Image Program (bottom panel). The experiment was repeated three times to identify the inhibitory concentration range, with similar results in each case. Results from a single representative experiment are shown. (**B**) The binding assays with GST-N-PH and unlabelled Bac1.2F5 cell lysate were repeated with either the maximal inhibitory concentration of p-Tyr (30 mM) compatible with the binding assay, or the same concentration of phosphothreonine (p-Thr). Migration of the 30 kDa protein, detected by Western blot with the anti-(p-Tyr) antibody, is indicated by the arrow.

with a lysate from ^{32}P -labelled Bac1.2F5 cells. A strong signal at 30 kDa was detected by autoradiography (Figure 3, left panel). This ^{32}P -labelled phosphoprotein co-migrated with [^{35}S]methionine-labelled 30 kDa protein run on the same gel as a marker (results not shown).

To determine whether the 30 kDa protein was phosphorylated on tyrosine residues, Western blot analysis was performed with the anti-(p-Tyr) antibody PY-20. In this experiment, the binding assay was repeated with an unlabelled Bac1.2F5 cell lysate and bound protein was subjected to SDS/PAGE and Western blot analysis with the anti-(p-Tyr) antibody. PY-20 antibodies bound to p-Tyr were detected with peroxidase-linked anti-mouse IgG antiserum, by using enhanced chemiluminescence. A strong signal was detected at 30 kDa (Figure 3, right panel), suggesting that the 30 kDa protein was a p-Tyr protein. Note that a weak signal was observed at the position of GST-N-PH fusion protein migration. This is not indicative of tyrosine phosphorylation but reflected the presence of the fusion protein used in the binding

assay, which frequently gave a non-specific signal because of its abundance.

Binding of the 30 kDa protein to N-PH is inhibited by free p-Tyr

To determine whether the p-Tyr residue of the 30 kDa protein itself directly contributes to the binding to N-PH, the ability of exogenous p-Tyr to inhibit binding was examined. The results showed that free p-Tyr inhibited the binding and that the inhibition was concentration-dependent (Figure 4). At a final concentration of 30 mM in the binding assay, p-Tyr inhibited approx. 75% of the binding signal detected by Western blot analysis (Figure 4A) (analysis was performed with the NIH Image Program). The concentration of p-Tyr required for effective inhibition was close to that reported in similar studies performed with the SH2 domain, another conserved protein motif that binds to tyrosine-phosphorylated peptides [41]. To ensure that the inhibition was specific for p-Tyr, phosphothreonine was added into the binding buffer at a final concentration of 30 mM. Phosphothreonine used at this concentration failed to inhibit the binding (Figure 4B).

Binding of the 30 kDa p-Tyr protein is specific for N-PH

To examine whether the binding was specific for N-PH, the binding assay was repeated instead with GST-C-PH. When equimolar amounts of the two fusion proteins were used, the binding of the 30 kDa protein to N-PH was significantly greater than that to C-PH (Figures 5A and 5B). Even when a 5-fold molar excess of GST-C-PH fusion protein was used in the binding assay, it still gave a weaker signal than 1 × GST-N-PH (Figure 5). This strong preference for N-PH suggests that there is specificity in the interaction between the 30 kDa p-Tyr protein and N-PH.

Lysine-14 and lysine-22 in the positively charged phosphoinositide-binding region of N-PH significantly contribute to binding

Two regions of the PH domain have emerged as likely candidates for mediating binding to p-Tyr residues (Figure 6A). First, the positively charged region at the entrance to the β -barrel, previously shown to be critical for the binding of phosphoinositide to PH domains, could also mediate binding to a negatively charged p-Tyr residue. Analysis of the amino acid sequence and the three-dimensional structure of N-PH [42], as well as of other PH domains for which binding has already been characterized, suggested to us that the positions of lysine residues 13, 14, 22 and 45 were all such that they might have the potential to interact with a negatively charged p-Tyr residue (see the Discussion section). Secondly, because PH domains are structurally similar to PTB domains, we examined the residues required for the binding of p-Tyr to IRS-1 and Shc PTB domains. A comparison of the three-dimensional structures showed that the two arginine residues responsible for the binding of p-Tyr to IRS-1 (arginine-212 and arginine-227) were located in approximately the same positions as two lysine residues in N-PH [see Figure 6(A) and the Discussion section]. If N-PH were binding to the 30 kDa p-Tyr protein with the same orientation as IRS-1 to its phosphopeptide ligand, it would be likely that these two residues, lysine-51 and lysine-75, would mediate the interaction. Although it is less clear, owing to the lower sequence and structural similarities between Shc PTB and N-PH, examination of the general orientation for binding of p-Tyr to Shc also implicated these same residues. To distinguish between these two possibilities and to identify the N-

Figure 5 Binding of the 30 kDa protein to both pleckstrin PH domains, but more strongly to N-PH than to C-PH

(A) Equimolar amounts of GST-N-PH, GST-DEP and GST-C-PH were used in the binding assays with unlabelled Bac1.2F5 cell lysates. The bound 30 kDa protein was detected with anti-(p-Tyr) antibody on a Western blot. In the lane marked GST-C-PH(5 ×), the assay was performed instead with a 5-fold molar excess of the GST-C-PH fusion protein. (B) A comparison of the extent of 30 kDa protein binding observed for the different assay conditions shown in (A). The analysis was performed on the data from (A) and from two additional repetitions of the binding assays by using the NIH Image program. * $P < 0.02$.

PH p-Tyr-binding site, lysine residues 13, 14, 22, 45, 51 and 75 were individually mutated to alanine residues (mutations referred to as K13A, K14A, K22A, K45A, K51A and K75A respectively). A double mutant, KK51/75AA, of the putative PTB-like p-Tyr-binding region was also prepared. The resulting mutant proteins were expressed as fusion proteins and used in the binding assay.

Results of the binding assay showed that mutations in the PTB-like p-Tyr-binding domain, NPH-K51A, NPH-K75A and NPH-KK51/75AA, had little impact on the strength of the binding signal observed with the wild-type N-PH protein (Figure 6B). In contrast, two of the mutations in the positively charged phosphoinositide-binding region significantly decreased the observed binding signal. Whereas the NPH-K45A mutation had a limited impact on binding, the NPH-K14A and NPH-K22A mutations each significantly decreased the strength of the binding signal (Figure 6C). This result suggests that lysine-14 and lysine-22 are important for binding. Mutation of lysine-13 (NPH-K13A) also repeatedly decreased, but did not abolish, binding. Thus, lysine-13 might also contribute to the ability of N-PH to bind to the 30 kDa protein. Results with these mutants suggest that the 30 kDa protein binds to N-PH through a site that includes or shares considerable overlap with the positively charged phosphoinositide-binding region of the PH domain.

Figure 6 Binding of the 30 kDa protein is decreased by point mutations of lysine residues on the positively charged surface of N-PH

(A) The approximate three-dimensional positions of the six lysine residues in N-PH that we mutated independently to alanine residues. Primers and procedures used to prepare the mutant constructs, and the expression and purification of the mutant GST fusion proteins, are described in the Experimental section. Residues 51 and 75 are located at positions analogous to those necessary for the binding of p-Tyr to the IRS-1 PTB domain (arginine-212 and arginine-227). Residues 13, 14, 22 and 45 are located in the region implicated in phosphoinositide binding. (B, C) Results of the binding assays with the 30 kDa protein to either the wild-type N-PH (NPH-WT) fusion protein or to equimolar amounts of the mutant N-PH fusion proteins. Unlabelled Bac1.2F5 cell lysates were used in these experiments; the intensity of the binding signal was detected by Western blot analysis with anti-(p-Tyr) antibody. In each case the result of a single representative experiment is shown. (B) The effect of mutation of either lysine-51 (NPH-K51A) or lysine-75 (NPH-K75A) or the double mutation of both residues (NPH-KK51/75AA), on the binding of the 30 kDa protein. (C) Left panel: the effect of mutation of either lysine-13 (NPH-K13A) or lysine-14 (NPH-K14A) on the binding of the 30 kDa protein. Right panel: the effect of mutation of either lysine-22 (NPH-K22A) or lysine-45 (NPH-K45A) on the binding of the 30 kDa protein.

DISCUSSION

Because the diversity in primary sequence among PH domains and the unique function of each PH domain suggested that the scope of potential ligands should be expanded, we chose to search for novel pleckstrin-binding partners by screening metabolically labelled haemopoietic cell lysates. Using a GST-N-PH fusion protein as probe, we consistently observed a 30 kDa

protein from ^{35}S -labelled lysates of both HL-60 and Bac1.2F5 cells. This protein was never observed in lysates probed instead with GST-DEP and was detected only faintly in lysates probed with the same concentration of GST-C-PH. The concentration of N-PH chosen for use in the binding assays, $3.5\text{ }\mu\text{M}$, was lower than the estimated intracellular concentration of pleckstrin in neutrophils ($15\text{ }\mu\text{M}$ [43]) so as to minimize non-specific binding.

The identity of our 30 kDa protein and its physiological role remain unknown but it is interesting that when platelet lysates or electroporated platelets were cross-linked with the bifunctional reagent bis(sulphosuccinimidyl)suberate, pleckstrin became cross-linked to an unidentified 28 kDa protein [44]. This suggests that pleckstrin and a 28 kDa protein exist in close proximity in platelets. Further identification of our 30 kDa protein is necessary to determine whether it is the same as the previously reported 28 kDa protein, and whether the 28 kDa protein and/or our 30 kDa protein are likely to interact with pleckstrin *in vivo*.

It is also interesting that two other proteins were detected in our N-PH-binding assays (Figure 2). A possible identity of the 43 kDa protein is the previously reported 40–45 kDa inositol-polyphosphate 5-phosphatase I that has been isolated from several tissues, including platelets [45]. This inositol 5-phosphatase co-immunoprecipitates with pleckstrin in platelet homogenates; phosphorylation of pleckstrin activates the bound inositol phosphatase severalfold [46]. A possible identity for the 80 kDa protein in our binding assays would be PKC, which has already been shown to bind to some PH domains [21,22]. The abilities of these proteins to bind to pleckstrin would be consistent with their known roles in phosphoinositide-mediated cellular signalling.

Our 30 kDa protein was also tyrosine-phosphorylated, as shown by ^{32}P labelling and by immunoblotting with anti-(p-Tyr) antibody. The ability of free p-Tyr to compete for binding suggested that the p-Tyr residue itself was critical for binding between the two proteins. A far-Western blot, with partly purified 30 kDa protein and anti-GST antibody to detect bound GST-N-PH fusion protein used as probe, showed that the binding itself was direct and did not require an intermediary protein or phosphoinositide (results not shown). This is the first report of a tyrosine-phosphorylated protein binding to a PH domain in which the phosphorylated residue seems to be critical to the binding event.

To determine which of the potential binding sites of N-PH was responsible for the 30 kDa protein binding, we examined the three-dimensional structure of N-PH obtained from the Brookhaven Protein Database (submitted by Yoon et al. [42]). Because we initially speculated that the region comparable to the p-Tyr-binding site of PTB domains was also the likely binding site for the 30 kDa protein, we examined the ligand-binding sites of the only two p-Tyr-binding PTB domains for which structural information was available, IRS-1 [26] and Shc [27]. Examination of the IRS-1 PTB domain showed that the peptide ligand fits in the cleft formed by the C-terminal α -helix and the β 5-strand. A pair of positively charged residues, arginine-212 and arginine-227, are positioned such that they interact directly with the phosphate group of the p-Tyr peptide ligand [26]. A comparison of this region with the N-PH structure showed a striking similarity and that, instead of a pair of arginine residues, a pair of lysine residues, lysine-51 and lysine-75, originated from approximately the same positions and with their positive side chains oriented in the same spatial direction. No other likely candidate residues were present in this region of the structure. A comparison of N-PH with the more divergent Shc p-Tyr-binding site [27] implicated the same two residues as candidates for mutagenesis.

We therefore prepared single point mutations of lysine-51 (K51A) and lysine-75 (K75A) and a double mutation of both residues (KK51/75AA) to test this possibility. When used in the binding assay, the mutant proteins did not alter the strength of the binding signal obtained with an equal molar concentration of wild-type N-PH. It was therefore unlikely that N-PH bound to the 30 kDa protein through its putative PTB-like p-Tyr site; we considered instead whether binding could be mediated through residues on the positively charged surface near the entry of the β -barrel. A structural similarity between the negatively charged inositol phosphate and the p-Tyr residue suggested the potential for overlap in their binding sites through this surface.

NMR structural studies have shown changes in the signalling patterns of lysine-13, lysine-14 and lysine-22 during PtdIns(4,5) P_2 binding to N-PH [6]. Lysine-13, lysine-14 and lysine-22 were located at positions comparable to those in phospholipase C δ_1 that are responsible for associating with the 4- and 5-phosphates of the inositol ring [7]. These three residues, along with lysine-45, were therefore subjected to mutagenesis. Lysine-45 is not conserved in other PH domains but its position and proximity to the other charged residues led us to consider the possibility of its also being involved with binding. Mutations of these residues would be expected to cause minimal structural change in the expressed proteins because the residues are exposed to solvent. When the mutant proteins were expressed in *E. coli*, their expression was comparable to that of the wild type. When equimolar amounts of these mutant proteins were used in the binding assay, a remarkable inhibition was observed with K14A and with K22A. Therefore our results suggest that the positively charged region of a PH domain might serve as a site for protein-protein as well as protein-lipid interaction.

It is not yet known whether our 30 kDa protein interacts with the pleckstrin PH domain *in vivo*. Even though N-PH was the first PH domain shown to be capable of binding to phosphoinositides [6], that binding proved to be of such low affinity ($K_d \approx 30 \mu\text{M}$) that it seemed insufficient to direct the membrane translocation of pleckstrin that occurs during cellular signalling [5]. This led us to search for additional ligands for N-PH, which has been shown to be the PH domain required for membrane translocation [47]. The potential interaction of pleckstrin with a tyrosine-phosphorylated protein is an intriguing possibility. Many tyrosine-phosphorylation events occur rapidly in platelets in response to thrombin or other agents that initiate aggregation and secretion, and also occur in other haemopoietic cells in response to agents that activate growth factor receptors, such as CSF-1, and lead to cellular signalling. Even if pleckstrin and the 30 kDa protein prove not to interact *in vivo*, the binding event characterized here might be indicative of interaction with a physiological ligand and needs further study. It is important to obtain the sequence of the p-Tyr peptide and to determine whether a common structural motif in the phosphorylated protein contributes to PH-domain binding. This information not only should clarify the binding partners of pleckstrin *in vivo* but also might reveal a common binding motif that could lead to the identification of the binding partners of other PH domains *in vivo*.

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