Selective cellular effects of overexpressed pleckstrin-homology domains that recognize PtdIns(3,4,5)P₃ suggest their interaction with protein binding partners

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Summary
Several pleckstrin-homology (PH) domains with the ability to bind phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P₃, PIP₃] were expressed as green fluorescent protein (GFP) fusion proteins to determine their effects on various cellular responses known to be activated by PIP₃. These proteins comprised the PH domains of Akt, ARNO, Btk or GRP1, and were found to show growth-factor-stimulated and wortmannin-sensitive translocation from the cytosol to the plasma membrane in several cell types, indicating their ability to recognize PIP₃. Remarkably, although overexpressed Akt-PH–GFP and Btk-PH–GFP were quite potent in antagonizing the PIP₃-mediated activation of the Akt protein kinase, such inhibition was not observed with the other PH domains. By contrast, expression of the PH domains of GRP1 and ARNO, but not of Akt or Btk, inhibited the attachment and spreading of freshly seeded cells to culture dishes. Activation of PLCγ by epidermal growth factor (EGF) was attenuated by the PH domains of GRP1, ARNO and Akt, but was significantly enhanced by the Btk PH domain. By following the kinetics of expression of the various GFP-fused PH domains for several days, only the PH domain of Akt showed a lipid-binding-dependent self-elimination, consistent with its interference with the anti-apoptotic Akt signaling pathway. Mutations of selective residues that do not directly participate in PIP₃ binding in the GRP1-PH and Akt-PH domain were able to reduce the dominant-negative effects of these constructs yet retain their lipid binding. These data suggest that interaction with and sequestration of PIP₃ may not be the sole mechanism by which PH domains interfere with cellular responses and that their interaction with other membrane components, most probably with proteins, allows a more specific participation in the regulation of specific signaling pathways.

Key words: PH domain, PI 3-kinase, PtdIns(3,4,5)P₃, PIP₃, Akt kinase, Cell adhesion, GFP

Introduction
Pleckstrin-homology (PH) domains are protein modules with a characteristic fold that have gained a great deal of interest as a result of their ability to bind phosphoinositides (Harlan et al., 1994; Lemmon et al., 1997; Lemmon, 2003). PH domains are present in a large variety of signaling molecules such as tyrosine or serine/threonine kinases, guanine nucleotide exchange factors and GTPase-activating proteins, phospholipases and a number of adaptor proteins (Lemmon, 2003). Some PH domains display high affinity and specificity towards specific isomers of inositol lipids, whereas others have lower affinity and show no clear preference for a particular phosphoinositide species (Yu et al., 2004). Several PH domains have been shown to be the main determinant of the recruitment of proteins to specific membrane compartments in a lipid-dependent manner, leading to the idea that PH domains serve as localization signals responding to local formation of inositol phospholipids (Lemmon and Ferguson, 2000). On the basis of these ideas, several PH domains, notably those of PLCγ1, Akt, Btk and GRP1 have been utilized successfully to monitor phosphoinositide changes in single living cells (Odorizzi et al., 2000; Balla and Varnai, 2002; Cozier et al., 2004).

Several observations suggest that PH domains might function in a more complex manner. In many proteins, such as in dynamin, the PH domain alone does not have high enough affinity to phosphoinositides to determine solely the localization of the protein, yet the protein shows clear regulation by inositol lipids (Artalejo et al., 1997; Szaszak et al., 2002; Yu et al., 2004). Moreover, using in vitro assay systems, it has been shown that the PH domains of Btk and PLCγ1 could transmit regulatory effects by the respective lipid, suggesting that PH domains might have conformational regulatory roles (Saito et al., 2001; Lomasney et al., 1996). Third, several PH domains are also capable of protein-protein interactions and in most cases the protein interaction surface does not overlap with the lipid-binding region within the PH domain (Carman et al., 2000; Lodowski et al., 2003). Also, in spite of their great success in providing invaluable spatial and
temporal information on inositide dynamics, the PH-GFP fusion proteins have certain limitations, mainly that they do not always report on the entire lipid pool that they are supposed to recognize (Balla et al., 2000; Irvine, 2004).

These observations prompted us to evaluate the principles of interactions of PH domains with the plasma membrane within the intact cells. We have chosen four PH domains with known ability to interact with membrane phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3, PIP_3] to determine whether their overexpression would affect selected PIP_3-regulated cellular functions in a similar manner. Our data show that the PH domains have distinctive inhibitory profiles on specific PIP_3-regulated pathways indicating that, in addition to binding to inositol lipids, these small protein modules could sequester additional specific protein components of particular signaling pathways.

Materials and Methods

Materials
Recombinant human EGF and platelet-derived growth factor (PDGF) were obtained from Life Technologies. Wortmannin and LY 294002 were purchased from Calbiochem. Myo-[^3H]inositol (68 Ci/mmol) was from Amersham-Pharmacia Biotech, and [^3H]inositol (1,3,4,5)-tetrakisphosphate (InsP_4; 22 Ci/mmol) was from Perkin Elmer. All other chemicals were of HPLC or analytical grade.

DNA constructs
The PH domains of Btk and Akt, as well as their mutants, have been described previously (Varnai et al., 1999; Servant et al., 2000). The human EGF receptor cDNA was subcloned from the SPER-7 plasmid, kindly provided by A. Clark and I. Pastan, NIH, Bethesda, MD, USA. (Clark et al., 1986), into the pCDNA3.1(−) plasmid (Invitrogen) with Hind(III) and HindIII restriction enzymes. The ARNO (239-399) and GRP1 (267-399 and 245-399) PH domains were amplified from marathon-ready human brain cDNAs (Clontech). The amplified DNA encoding the GRP1 PH domain was cloned between the BglII/EcorI sites and the ARNO PH domain between the EcoRI/BamHI sites of the pEGFP-C1 plasmid. Mutations within the PH domains were generated by the Quikclone mutagenesis kit (Stratagene). All constructs were confirmed by dideoxy sequencing. Recombinant PH domains were created by subcloning the exact same constructs used for the mammalian expression studies (including the mutants) into the pET-23b plasmids containing the 6×His residues at the C-terminus.

Akt activity measurements
COS-7 cells were seeded onto 35 mm culture dishes and transfected with HA-Akt (Bondeva et al., 1998) and the indicated plasmid DNA constructs (1 µg each) at 60% confluence using the lipofectamine 2000 reagent. 24-36 hours after transfection, cells were lysed in 1 ml lysis buffer containing 50 mM Tris/Hcl, pH 7.5, 150 mM NaCl, 1 mM Na-pyrophosphate, 1% NP-40, 20 mM β-glycerophosphate, 10% glycerol and protease inhibitors (0.1 mM Na_2VO_4, 25 mM NaF, 1 µg/ml aproninin, 0.02 mM leupeptin, 0.02 mM pepstatin and 100 µM AEBMSF) and 1 mM DTT freshly added prior to use. Lysates were cleared by centrifugation and 200 µl was incubated with 1 µg of anti-HA monoclonal antibody (Covance) for 1-2 hours at 4°C before the addition of 15 µl protein G- sepharose prewashed in lysis buffer for an additional incubation at 30 minutes. Immunoprecipitates were washed twice with lysis buffer, once with 100 mM Tris/0.5 M LiCl pH 7.5, and finally with the kinase reaction buffer without ATP. Beads were then resuspended in 50 µl kinase buffer (20 mM Heps, pH 7.5, 10 mM MgCl_2, 1 mM DTT) containing 2 µCi γ-[^32P]ATP, 10 µM ATP and 30 µM crosstide peptide. Reactions were run for 30 minutes at 30°C and terminated by rapid centrifugation (9000 g/5 minutes) and transfer of 40 µl reaction product to a Whatman P81 phosphocellulose filter. Filters were washed four times with 75 mM phosphoric acid and once in acetone before drying and scintillation counting. Expression levels of the various components were tested in each experiment from the lysates by western blot analysis. In later experiments, endogenous Akt activation was measured by western blotting using the phosho-Akt (Ser473) antibody (Cell Signaling) and the data were analyzed with densitometry.

Transfection of cells for confocal microscopy
Cells were plated onto 25 mm diameter circular glass cover slips at a density of 3×10^5 cells/dish and were transfected the next day with plasmid DNAs (2 µg/ml) using the Lipofectamine 2000 reagent and OPTI-MEM (Life Technologies). 24-36 hours after transfection, cells were washed twice with a modified Krebs-Ringer buffer, containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl_2, 0.7 mM MgSO_4, 10 mM glucose, 10 mM Na-Hepes, pH 7.4, and the coverslip was placed into a chamber that was mounted on a heated stage with the medium temperature kept at 33°C. Cells were incubated in 1 ml of the Krebs-Ringer buffer and stimuli were added in 0.5 ml prewarmed buffer after removing 0.5 ml medium from the cells. Cells were examined in an inverted microscope under a 40× oil-immersion objective (Nikon) and a BioRad laser confocal microscope system (MRC-1024) with the Lasersharp aquisition software (BioRad) or in a Zeiss LSM510 laser confocal microscope.

Cell attachment assay
COS-7 cells (2×10^5) were plated onto 10 cm culture dishes and transfected the next day using Lipofectamine 2000 (5 µg DNA/dish). After 24-36 hours, cells were removed from the plates by gentle trypsinization and divided into three aliquots. One aliquot was centrifuged and the cells were immediately lysed in Laemmli buffer. The other two aliquots were plated into 35 mm culture dishes and the cells were allowed to attach for 30 minutes. After this, the medium was removed and the cells were washed twice with 2 ml of ice-cold Dulbecco’s phosphate-buffered saline (PBS) before lysis in the same volume of Laemmli buffer that was added to the first aliquot of cells. Samples were then sonicated (but not boiled) and loaded onto a 8-16% Tris/glicerine gel and separated by SDS-PAGE. Electrophoresis gels were analyzed in a Storm 860 Phosphorimagger (Molecular Dynamics) using the blue fluorescent laser for quantitation of the GFP fusion protein band in the gel. The fraction of GFP signal found in the attached cells relative to the total amount of cells seeded was calculated for each construct as well as GFP alone, which served as a control.

Cell spreading on fibronectin
Glass coverslips were coated with fibronectin (20 µg/ml) for 2 hours at 37°C, and washed twice with PBS to remove any unbound fibronectin. To block any remaining binding sites, coverslips were then incubated with 1 mg/ml fatty acid free bovine serum albumin (BSA) for 1 hour at 37°C. COS-7 cells expressing the different PH domain constructs (24 hours after transfection) were prepared for plating by trypsinization. After pelleting, cells were treated with 0.3 mg/ml soybean trypsin inhibitor in DMEM for 10 minutes at 37°C followed by incubation in 0.5% BSA in DMEM for an additional 1 hour to allow cell recovery. Cells were then plated onto the fibronectin-coated coverslips and fixed by 4% paraformaldehyde 10 minutes after plating. Fixed cells were permeabilized by 0.2% Triton X-100 in PBS for 5 minutes followed by phallolidin staining (0.1 µg/ml TRITC-phallolidin in PBS for 20 minutes). Cells overexpressing the GFP fusion proteins were identified by fluorescent
microscopy without knowing the treatment regimen and were classified into three groups: unspread (adherent with no projections), partially spread (adherent with limited lamellipodia), and fully spread. The percent of fully spread cells was then calculated for each group of cells expressing the GFP fusion proteins.

Analysis of expression kinetics
To transfect COS-7 cells in 96-well plates (black with clear bottom) 1 μg DNA in 25 μl OPTI-MEM/well were mixed with 0.5 μl Lipofectamine-2000 in 25 μl OPTI-MEM/well and incubated for 30 minutes in the well. Freshly trypsinized COS-7 cells (4.5×10^3/100 μl OPTI-MEM/well) were then added to the wells and the plate was incubated in a CO2 incubator. After 6-10 hours, 150 μl/well DMEM containing 10% FBS was added. GFP fluorescence was measured at the indicated times using a plate reader (Ascent FL, ThermoLabsystems; or Mithras LB940, Berthold). To determine the PIP3-dependent component of the effects of the expressed PH domains on the balance of proliferation/apoptosis, the ratios of the fluorescence of cells expressing the wild-type PH domains were calculated using the mutant forms of the same domain incapable of lipid binding as a control at each time point for each PH domain.

Analysis of inositol phosphates
Inositol phosphates were analyzed from COS-7 cells transfected with cDNA encoding the human EGF receptor, together with selected PH-Inositol phosphates were analyzed from COS-7 cells transfected with cDNA encoding the human EGF receptor, together with selected PH-domains on the balance of proliferation/apoptosis, the ratios of the fluorescence of cells expressing the wild-type PH domains were calculated using the mutant forms of the same domain incapable of lipid binding as a control at each time point for each PH domain.

Protein purification and InsP2 or PIP3 binding
Recombinant PH-GFP proteins were produced in BL-21 cells (Invitrogen). Overnight cultures were grown to OD600: 0.6-0.9 and induced with 200 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 7 hours at room temperature. Proteins were purified from the bacterial lysates by Ni-NTA columns (Spin Kit, Qiagen) following the manufacturers instruction. InsP2 binding was performed as described previously (Varnai et al., 2002), using 1.1 nCi (1 nM) [3H]InsP2 and 200 ng proteins in the 50 μl incubation volume. Binding to PIP3-coated agarose beads (Echelon) was performed following the manufacturer’s protocol with minor modifications. To elute the proteins bound to the beads, after centrifugation and washing, beads were incubated at room temperature for 30 minutes instead of boiling to preserve fluorescence. The amounts of bound and free proteins were assessed by phosphoimager analysis of the samples resolved by SDS PAGE.

Results
Recognition of PIP3 by the PH domains in NIH 3T3 cells
Four PH domains known to interact with PIP3 were chosen for this study: Btk-PH, Akt-PH, GRP1-PH and ARNO-PH. Since the ARNO-PH domain has two splice variants, differing only in a single glycine in the loop between the β1- and β2-strands (Klarlund et al., 2000; Lietzke et al., 2000) that changes the lipid binding specificity of the domain, we also included the 3G variant (ARNO-3G) in these studies. As documented by several studies, all of these proteins show a PIP3-dependent translocation to the plasma membrane in many cell types after agonist stimulation (Venkateswarlu et al., 1998; Oatey et al., 1999; Varnai et al., 1999; Watton and Downward, 1999). As shown in Fig. 1, clear differences are found in the distributions of these proteins in quiescent NIH 3T3 cells, namely the prominent nuclear localization of GRP1-PH and ARNO-PH, which was much less pronounced with Btk-PH and very little with Akt-PH. Also, a small extent of plasma membrane localization was observed with Akt-PH, in quiescent cells but not with the other PH domains. However, all of the chimeras showed a robust translocation to the plasma membrane after PDGF stimulation that was completely reversed by wortmannin in the case of Btk-PH, GRP1-PH and ARNO-PH, and was largely, but not completely, eliminated in the case of Akt-PH–GFP. The ARNO-PH domain variant containing three

Fig. 1. Localization and wortmannin-sensitive translocation of expressed PH-GFP chimeras that recognize PIP3 in NIH 3T3 cells. NIH 3T3 cells were transfected with the indicated PH-GFP constructs and live cells were examined by confocal microscopy after 1 day of transfection. Note the recruitment of the PH domains (except that of the 3G variant ARNO) to the plasma membrane after PDGF stimulation, and the reversal of this by the PI 3-kinase inhibitor wortmannin.
glycines showed little basal localization and a barely detectable translocation response. These data together demonstrated that the selected PH domains are able to recognize PIP3 in the plasma membrane with sufficiently high affinity.

Inhibition of Akt activation by the overexpressed PH domains

One of the best known and most studied downstream targets of PIP3 [and phosphatidylinositol (3,4)-bisphosphate, PtdIns(3,4)P2] is the serine/threonine kinase Akt. Therefore, first we decided to study the inhibitory effects of the expressed PH domains on Akt activation in COS-7 cells. In order to investigate the Akt response of only the transfected cells, an epitope-tagged Akt (HA-Akt) was transfected together with the selected PH-GFP constructs and also with a membrane-targeted form of phosphoinositide 3-kinase γ (PI3Kγ-CAAX) (Bondeva et al., 1998), to generate PIP3. After 24 hours of transfection, Akt was immunoprecipitated from the cell lysates and its activity was determined using 32P-ATP and crosstide as substrates (Bondeva et al., 1998). As shown in Fig. 2, membrane-targeted PI3Kγ strongly stimulated Akt activity and both Akt-PH and Btk-PH were able to inhibit this activation (P<0.05). By contrast, GRP1-PH and ARNO-PH had no effect. Mutant Btk-PH (R28C), which is unable to bind the lipid, was also without effect, indicating the need for lipid binding to exert an inhibitory effect.

Inhibition of cell attachment and spreading by the various PH domains

To test a PIP3-regulated process that is linked to the function of the GRP1/ARNO family of GTP-binding protein exchange factors, we chose two simple assays that rely upon inside-out signaling. Cell adhesion and spreading have both been shown to be PIP3-regulated processes in which integrins and small GTP-binding proteins have been implicated (Kinashi et al., 1995; Heraud et al., 1998; Yamboliev et al., 2001; Hawadle et al., 2002). COS-7 cells were transfected with the various GFP fusion constructs and were removed from the culture plates with Versene. Aliquots of the transfected cells were then reseeded onto small culture dishes and incubated for the indicated times (usually 30 minutes). Non-attached cells were then washed away and the attached cells were subjected to SDS page analysis along with the sample of the total amount of cells seeded, so that the fraction of transfected cells attached could be calculated after quantification of the GFP fluorescence in a Phosphorimager. This method allowed monitoring of the attachment efficiency of only the population of transfected cells. As shown in Fig. 3, the PH domains of GRP1 and ARNO were found to inhibit the attachment of cells (P<0.05), whereas the PH domains of Akt and Btk were without effect in this assay.

To examine the effect on cell spreading, transfected cells were also seeded on fibronectin-coated cover slips and incubated for 10 minutes before fixation and staining with TRITC-phalloidin. Cells were then examined under a wide-field fluorescence microscope and scored for the presence of spreading and/or lamellipodia. These data also showed that both GRP1-PH and ARNO-PH, but not Btk-PH or Akt-PH, exerted a strong effect on cell spreading (P<0.05, Fig. 4). Interestingly, ARNO-3G was also effective in these assays, although less so than the 2G variant.

Effect of PH domain expression on PLCγ-mediated inositol phosphate production

It has been well documented that PLCγ activation is partially dependent on PI3γ formation (Falasca et al., 1998; Bae et al., 1998). Therefore, we investigated whether the overexpressed PH domains exert an inhibitory effect on EGF-stimulated inositol phosphate production. COS-7 cells were transfected with the EGF receptor along with the various PH-GFP chimeras and were prelabeled with myo-[3H]inositol. EGF-stimulated inositol phosphate production was then tested in the presence of lithium to capture PLCγ-generated inositol phosphates. As shown in Fig. 5, about 40% of the EGF-stimulated InsP response (which was about twofold on average) was wortmannin sensitive. The EGF-induced response was also inhibited by the GRP1- and ARNO-PH domains, and to a lesser degree by the Akt-PH (P<0.05 in all cases). However, Btk-PH had a significant positive effect on this response that was reversed by wortmannin.

**Fig. 2.** Inhibition of PI3γ-dependent Akt activation by the various PH domains. COS-7 cells were transfected with a HA-tagged full-length Akt construct with or without a construct encoding a plasma-membrane-targeted PI3-kinase γ (PI3Kγ-CAAX) and the indicated PH-GFP constructs. One day after transfection and after 6-8 hours of serum deprivation, cells were lysed and the activity of HA-Akt was measured after immunoprecipitation as detailed under Materials and Methods. Equal expression of HA-Akt, as well as the other expressed proteins, was ensured by keeping the total transfected DNA equal by complementing with the respective empty plasmid DNAs during transfection and were determined from the total cell lysates. In each experiment, the Akt activities were normalized to the level observed with PI3Kγ-CAAX in the presence of GFP alone, which gave an average of tenfold increase over the basal. Mean±s.e.m. of three similar observations are shown.
Inhibition by overexpressed PH domains

Inhibition by overexpressed PH domains overexpression of complex cellular responses

In subsequent experiments, we investigated the abilities of the various PH domains to influence more-complex cellular responses such as proliferation and apoptosis. These responses rely upon a number of signal transduction events and therefore are more difficult to interpret in terms of the specific

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**Fig. 3.** Inhibition of cell attachment by overexpressed PH-GFP chimeras in COS-7 cells. COS-7 cells were transfected with the indicated PH-GFP constructs for 1 day. Cells were removed by mild trypsinization and divided into three aliquots. One aliquot was centrifuged and the cells were immediately lysed in Laemmli buffer (labeled c). The other two aliquots were plated into 35mm culture dishes and the cells were allowed to attach for 30 minutes (labeled a). After this, the medium was removed and the cells were washed twice with 2 ml of ice-cold Dulbecco’s PBS before lysis in the same volume of Laemmli buffer that was added to the first aliquot of cells. Samples were then sonicated (but not boiled) before separation by SDS-PAGE. Electrophoresis gels were analyzed in a Storm 860 Phosphorimager (Molecular Dynamics) using the blue fluorescent laser for quantitation of the GFP fusion protein band in the gel. The fraction of GFP signal found in the attached cells relative to the total amount of cells seeded was calculated for each construct including GFP alone, which served as a control. Representative gel samples are shown in (A), and the mean±e.m. from five experiments performed in duplicates are shown in (B).

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**Fig. 4.** Inhibition of cell spreading by overexpressed PH domain chimeras in COS-7 cells. COS-7 cells transfected with the indicated PH-GFP constructs for 24 hours were removed from the culture dishes with gentle trypsinization and re-plated onto fibronectin-coated glass cover slips as detailed under Materials and Methods. After 10 minutes, cells were fixed and stained with TRITC-phalloidin. Cells overexpressing the GFP fusion proteins were identified by fluorescent microscopy and were classified into three groups: unspread (adherent with no projections), partially spread (adherent with limited lamellipodia) and fully spread. The percentage of fully spread cells was then calculated for each group of cells expressing the GFP fusion proteins and related to the untransfected controls. When added, the PI 3-kinase inhibitor, LY 424002 (Ly) was added 10 minutes before plating on fibronectin. Mean±s.e.m. of 3-6 experiments are shown.
biochemical event being targeted; nevertheless, they could
reveal significant phenotypic changes caused by the
expression of PH domains. Therefore, we studied how the
expression of the fluorescent PH domain changes in time for
the individual constructs. Although the expression kinetic of
any given protein can vary as a result of multiple factors within
the cells, a difference between the expression dynamics of
wild-type and mutant forms (that do not bind
phosphoinositides) of the PH domains would indicate the
involvement of a PIP3-dependent process. For example, any
positive effect of the PH domain protein on apoptosis would
be expected to eliminate the transfected cells more rapidly and
hence rapidly decrease the population of fluorescent cells after
the construct is expressed. As shown in Fig. 6, the various
constructs showed significant variations in their expression
patterns. When the data were expressed as fluorescence of
wild-type version relative to the mutant version of the same
PH domain, the only remarkable difference was observed with
the Akt-PH domain, which clearly showed a lipid-binding-
dependent self-elimination.

Mutagenesis of Akt-PH and GRP1-PH domains
The specific inhibitory effect of the PH domains on selected
cellular responses raised the possibility that these domains might
interact with protein-binding partners in addition to binding
PIPs. The putative binding partners would have to interact with
a surface on the PH domain that is distinct from that involved
in lipid binding. It has been previously reported that Thr34 of the
Akt-PH domain is phosphorylated by PKCζ and this
phosphorylation prevents Akt membrane recruitment and
activation (Powell et al., 2003). This Thr residue is located on
the surface of the Akt-PH domains that is not directly involved
in PIP3 binding and which corresponds to the βγ-binding
interface of the GRK2-PH domain (Lodowski et al., 2003).
Therefore, we generated a series of Thr34 mutants with various
substitutions (T34A, T34S, T34P, T34D, T34L and T34F) within

Fig. 5. Inhibition of EGF-stimulated InsP formation by
overexpressed PH domain chimeras in COS-7 cells. COS-7 cells
were transfected with cDNA encoding the human EGF receptor,
together with selected GFP-PH domain fusion constructs as
described under Materials and Methods. One day after transfection,
cells were labeled with myo-[3H]inositol for 24 hours. Cells were
stimulated by EGF for 30 minutes in the presence of 10 mM LiCl,
and [3H]-labeled inositol phosphates were separated by Dowex
minicolumns and measured by liquid scintillation counting. The
relatively small activation of PLC by EGF in these cells did not
permit direct analysis of the 1,4,5-isomer of InsP3 and the pooled
InsP1 and InsP2 samples were used as an indicator of overall PLC
activity. The InsP response of the cells was normalized to the value
observed after EGF stimulation of cells expressing only GFP, which
was about a twofold increase in average. The PI 3-kinase inhibitor
wortmannin (wm) was added 10 minutes before EGF treatment.
Means±s.e.m. of 3-5 experiments are shown performed in duplicate
(except for Akt-PH, where n=2).

Fig. 6. The dynamics of expression of various PH-GFP chimeras in
COS-7 cells. COS-7 cells were transfected with the indicated PH-
GFP constructs and the GFP fluorescence was monitored as a
function of time using a fluorescence plate reader. Fluorescence
values were normalized to the first reading in each group (20 hours
after transfection) as shown for a representative experiment in (A).
To determine the PIP3-dependent component of the effects of the
expressed PH domains on the balance of proliferation/apoptosis, the
ratios of the fluorescence of cells expressing the wild-type PH
domains were calculated using the mutant forms of the same domain,
which are incapable of lipid binding, as a control at each time point
for each PH domain (B). Means±s.e.m., n=3.
Inhibition by overexpressed PH domains

the Akt-PH–GFP construct and determined their ability to bind PIP₃, localize to membranes in intact cells, and exert inhibition on Akt activation. Of these mutants, T34A and T34S behaved as the wild-type version, whereas T34P showed no membrane localization or binding to lipids (not shown). However, T34D, T34L and T34F displayed quite interesting features. As shown in Fig. 7, these mutants all showed prominent membrane localization in stimulated COS-7 cells or in HEK 293 cells, and only T34F showed a somewhat impaired membrane recruitment (Fig. 7A). This was in good agreement with the PIP₃ binding data, which showed that binding of these mutant recombinant proteins to PIP₃ beads was only slightly impaired (Fig. 7B). However, in spite of their PIP₃ binding and membrane localization, these mutants showed no inhibition on Akt activation compared with the R25C mutant that is unable to bind the lipids and localize to the membrane (Fig. 7C). Similarly, none of the Akt-PH mutants showed self-elimination in the assay monitoring the kinetics of expression of the domains as a function of time, in fact the T34F mutant showed better expression than the R25C mutant (panel D). These data clearly suggested that membrane localization is necessary but may not be sufficient for the construct to interfere with the signaling of the endogenous Akt protein, and the latter is related to a feature of the PH domain consistent with additional interaction(s).

Similar experiments were designed with the GRP1-PH domain. Here, two mutants were generated at residues located on the same surface probed with the Akt-PH domain, away from the lipid-binding site. The two mutants, I307E and K340L, were chosen because of their size and side chain orientation. As shown in Fig. 8, these mutations failed to affect the binding of the domain to PIP₃ beads (Fig. 8B); however, they did impair the abilities of the constructs to localize to the membranes of HEK 293 cells, although still showed localization in peroxystanadate-stimulated COS-7 cells (Fig. 8A). Because of their somewhat impaired cellular localization, the InsP₄-binding affinity of these constructs was also determined (Fig. 8C). These data showed no significant difference between the domains confirming that their PIP₃/InsP₄ affinities are not affected by the mutations. Importantly, neither mutant showed the inhibitory effect on cell spreading that was observed with the wild-type GRP1-PH domain, and their effect was less or equal to the R284C mutant unable to bind PIP₃ or localize to the membrane. These data also indicated that the dominant-negative effect of the GRP1-PH domain requires interactions other than with the lipid PIP₃.

Fig. 7. The effects of Akt-PH–GFP mutations on PIP₃ binding, cellular localization and inhibition of cellular responses. (A) COS-7 (upper) and HEK 293 cells (lower) were transfected with the indicated mutant Akt-PH–GFP construct and examined by confocal microscopy without stimulation (HEK 293 cells) or after stimulation with peroxystanadate (30 μM peroxide, 100 μM ortho-vanadate) for 5-10 minutes (COS-7 cells). (B) Binding of recombinant Akt-PH–GFP mutants to PIP₃ conjugated to agarose beads (sn, unbound fraction in the supernatant; pellet, bound fraction associated with the beads); bars represent the % bound fraction determined by Phosphorimager analysis from three separate experiments (±s.e.m.), one of which is shown as a representative. (C) Inhibition of endogenous Akt activation by Akt-PH–GFP mutants expressed in COS-7 cells. After 24 hours of transfection, cells were stimulated by EGF (100 ng/ml) for 5 minutes. Total cell lysates were analysed by SDS PAGE followed by western blotting using an anti-phospho-Akt antibody and densitometric analysis. In (B) and (C), the R25C mutant, unable to bind the lipid, is indicated by darker columns. (D) Expression kinetics of the various Akt-PH–GFP mutants in COS-7 cells as described in Fig. 6. Fluorescence values were related to those of the non-binding R25C mutant at each time points (mean±s.e.m., n=3). (E) The position of the mutated residue (Thr34) within the Akt-PH domain relative to the PIP₃ binding site in the crystal structure of Akt-PH (1H10).
Discussion

There is hardly a cellular process that is not regulated by phosphoinositides, and PI 3-kinases have been shown to affect a great variety of cellular responses. This notion has generated enormous interest in the downstream effectors of the 3-phosphorylated lipid product, PIP3, leading to the identification of numerous proteins that possess PH domains with specific PIP3 recognition properties (Klarlund et al., 1997; Welch et al., 2002). Although many of these PH domains bind PIP3 with high enough affinity that is sufficient to recruit them to the plasma membrane upon activation of PI 3-kinases, a number of observations suggest that they also interact with protein components. For example, the Btk- and GRK2-PH domains were shown to bind βy subunits of heterotrimeric G proteins (Tsukada et al., 1994; Carman et al., 2000), and Btk-PH also binds the C1 domain of PKC (Yao et al., 1994), whereas its PH/Tec-homology (TH) domain binds the mouse PIP 5-kinase type-Ιβ (Saito et al., 2003). Similarly, the Akt-PH was reported to interact with the myosin II isoform (Tanaka et al., 1999), although it is not clear whether this protein interaction contributes to the physiological regulation of the Akt protein kinase. Several other PH domains show relatively low specificity in their inositol lipid binding in vitro, yet appear to be regulated more specifically by the lipids within the cell (Yu et al., 2004). Therefore, an increasing body of evidence indicates that PH domains might serve as more complex molecular modules with both lipid and protein recognition.

The present experiments were designed to investigate whether PH domains that recognize the same phosphoinositide species, namely PIP3, exert similar inhibitory effect on distinct cellular processes known to be regulated by this critically important phosphoinositide. We reasoned that if PH domains solely interacted with the membrane lipids, their inhibitory potencies should follow an identical rank order determined by their relative lipid affinities regardless of the PIP3-dependent regulatory pathway examined. Our data clearly demonstrate that, of the selected PH domains with PIP3 recognition, some can quite specifically inhibit one PIP3-regulated process without significantly affecting another. For example, expression of the GRP1-PH domain was effective at inhibiting cell adhesion and spreading, but had no effect on Akt activation, whereas the Akt-PH domain behaved the opposite way, i.e. showing a strong inhibitory effect on Akt activation but no effect on the cell spreading process. These findings cannot be simply explained by sequestration of the lipids by the PH domains. In fact, inhibition by lipid sequestration may not be as easily achieved since production of the lipid can simply make up for the PH-domain-bound fraction in any given PIP3-mediated process. However, if PH domains also bind to protein partners that are important for a specific downstream signaling process, sequestration of this protein will not be easily compensated for, thereby explaining the

Fig. 8. The effects of GRP1-PH–GFP mutations on PIP3 or InsP4 binding, cellular localization and inhibition of cellular spreading in COS-7 cells. (A) COS-7 (upper) and HEK 293 cells (lower) were transfected with the indicated mutant GRP1-PH–GFP construct and examined by confocal microscopy without stimulation (HEK 293 cells) or after stimulation with peroxvanadate (30 μM peroxide, 100 μM ortho-vanadate) for 5-10 minutes (COS-7 cells). (B) Binding of recombinant GRP1-PH–GFP mutants to PIP3 conjugated to agarose beads (sn, unbound fraction in the supernatant; pellet, bound fraction associated with the beads); bars represent the % bound fraction determined by Phosphorimager analysis from two separate experiments (mean±range), one of which is shown as a representative. (C) Binding of InsP4 to GRP1-PH–GFP mutants. Recombinant proteins were incubated with [3H]InsP4 in the presence of increasing concentration of unlabeled InsP4 for 10 minutes. Protein-bound radioactivity was determined as described under Materials and Methods and expressed as % B0 (means±s.e.m., n=3). (D) Inhibition of cell spreading by the various GRP1-PH–GFP constructs as described in Fig. 4. (means±s.e.m., n=3). In (B) and (D), the R284C mutant, unable to bind the lipid, is indicated by darker columns. (E) The position of the mutated residues (I307 and K340) within the GRP1-PH domain relative to the PIP3 binding site in the crystal structure of GRP1-PH (1FHX).
selective inhibition of a particular pathway by the overexpressed PH domain.

Thus, mutations generated on a surface within the Akt-PH and GRP1-PH domains that is not directly involved in lipid binding can abrogate the inhibitory effects of the constructs without affecting lipid binding. This finding is also consistent with interaction of the domains with additional binding partners, most probably with other proteins. Also, a putative protein interaction with the PH domain might indirectly affect the membrane localization of the construct. This was seen more prominently with the GRP1-PH domain, which showed impaired membrane localization in spite of an unchanged lipid-binding affinity, whereas the selected Akt-PH domain mutants still localized to the membrane. It is worth pointing out that C2 ceramide has been reported to eliminate the binding of both Akt-PH–GFP and GRP1-PH–GFP after PDGF stimulation (Stratford et al., 2001), an affect attributed to the PKCζ-mediated phosphorylation of T34 in the case of the Akt-PH domain (Powell et al., 2003). In the present study, the T34D substitution failed to mimic the phosphorylation effect on membrane localization, but this could be due to the much larger charge of the phosphate residue in this position.

It is important to note that the selectivity of the PH domains was not absolute, for example, the Btk-PH also inhibited Akt activation and all but Btk-PH was able to inhibit EGF-induced InsP₃ formation. Whether this reflects a similarity between the domains in regard to the protein interacting partner, or the multiple components through which InsP₃ generation can be affected in the latter case, remains to be elucidated. It is also important to emphasize that, in the case of responses regulated by more complex regulatory networks, such as the growth of the transfected cells, multiple effects could determine the final outcome. For example, although Btk-PH inhibited Akt activation similarly to that of Akt-PH, the two domains affected the EGF-mediated InsP₃ response differently, Akt-PH having an inhibitory effect, and Btk-PH having a strong stimulatory effect. As a result, only Akt-PH caused a selective elimination of the cells in which it was expressed; by contrast, in the case of Btk, the two opposing effects seem to have balanced one another and hence, Btk-PH had no prominent effect on cell elimination.

These findings have important implications regarding inositol lipid regulation of effectors containing PH domains. It is conceivable that, upon lipid binding, the PH domain undergoes a conformational change that will affect its interaction with a protein-binding partner. This partner could be either another regulatory protein or another domain within the same protein forming an intramolecular interaction. A conformational change upon binding of InsP₄ to the Akt-PH has been described recently (Milburn et al., 2003) but not many other studies are available in which this question has been examined at the structural level. By contrast, there are several examples of PH domains regulating the functions of proteins; this has been observed even in vitro, where PIP₃-mediated membrane recruitment cannot be responsible for the observed effects of the lipid. For example, Btk kinase activity in vitro was found to be stimulated by PIP₃ (Saito et al., 2001). Recently, the PH domain of the ELMO protein (which, together with Dock180, functions as a bipartite Rac guanine-nucleotide exchange factor) was shown to be essential for the binding and regulation of the Rac-Dock180 complex independent of membrane targeting (Lu et al., 2004). Similarly, in several guanine nucleotide exchange factors with tandem Dbl-homology (DH) and PH domains, the PH domain may cooperate with the DH domain in promoting nucleotide exchange (Rossman et al., 2002; Soisson et al., 1998; Worthylake et al., 2004), although this may not be regulated by inositides in vitro (Snyder et al., 2001). Conversely, the inositol lipid binding of the nucleotide exchange factor Trio was found to be influenced by the binding of RhoG (Skowronek et al., 2004), providing additional support to the idea that the lipid and protein binding of PH domains might regulate one another.

In summary, the present studies demonstrate that overexpression of isolated PH domains can act as inhibitors of PIP₃-regulated cellular pathways and show a degree of specificity that was unexpected given their inositol lipid recognition properties. Selected mutations that do not affect their lipid recognition are able to prevent the inhibitory effects, suggesting that PH domains might also interact with proteins. PH domains, therefore, could serve as molecular switches regulated by phosphoinositides. This type of regulation could explain the parallel and specific control of multiple effectors by the membrane phospholipids. More studies will be needed to validate this concept and to identify the protein regulatory partners that participate in the process.

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