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13. ABSTRACT (Maximum 200 Words)

We are developing an *in vivo* system using erbB/IL2 receptor chimerae in a B-cell line to investigate the interactions and mechanism of oligomerization between the epidermal growth factor (EGF) receptor family members erbB1 and erbB2. Since erbB2 overexpression has been strongly associated with breast cancer and has been shown to be a valuable target for breast cancer therapies, we are interested in dissecting its mechanism of activation. Heteromeric interaction between the intracellular domains of the IL2 receptor beta and gamma chain will serve as a reporter for direct interaction between the extracellular domains of erbB1 and erbB2 by mediating T or B-cell proliferation in the absence of IL2. Previously, we have demonstrated erbB1 homo-oligomerization and hetero-oligomerization with erbB2 in an EGF dependent manner. Due to problems in generating required cell lines expressing multiple chimeric receptors, we have not been able to address the remaining aims and have recently focused considerable effort to this end with little success. With further work, we hope to fully develop this assay to understand how erbB1 and erbB2 interact and to provide insight into the mechanism by which erbB2 mediates transformation and tumorigenicity in cells.

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Introduction

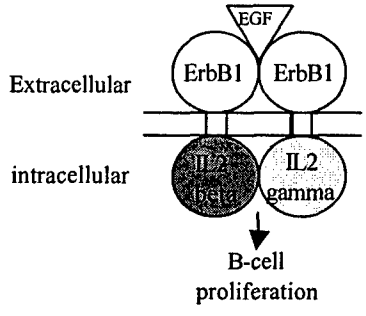
The epidermal growth factor (EGF) receptor family members (consisting of erbB1, erbB2, erbB3, and erbB4) are tyrosine kinase receptors which transduce extracellular signals by ligand mediated receptor homo- and/or hetero-oligomerization and subsequent transphosphorylation of the opposing oligomer member. This results in the recruitment of specific SH2 or PTB containing proteins leading to the activation of various signaling pathways. ErbB2, which possesses no known ligand, is thought to activate signaling pathways only through hetero-oligomerization with the other receptor members. ErbB2 has been shown to play a role in breast cancer, as amplification and overexpression of this gene are observed in a significant fraction of human breast cancers. Furthermore, high levels of erbB2 expression correlate with an aggressive tumor phenotype and poor patient prognosis. With the inception of Herceptin, a humanized antibody directed against erbB2, it has become clear that erbB2 may be an important target for breast cancer treatment. While it is unclear how erbB2 causes transformation and tumor formation, current evidence suggests that ligand dependent hetero-oligomerization may play a primary role. In our studies, we hope to dissect the mechanism of erbB2 hetero-oligomerization (and subsequent activation) with erbB1. We planned to achieve this by developing an *in vivo* reporter system using erbB/IL2 receptor chimerae, where ligand mediated oligomerization of the extracellular domains of the erbB receptors will in turn drive oligomerization of the IL2 receptor intracellular beta and gamma chains of these receptor chimerae. This intracellular beta-gamma chain association will generate a proliferative signal in B or T cells (which are erbB receptor null) in an IL2 independent manner. With further work and development of this approach, we hope to dissect and understand how erbB2 is normally activated and suggest novel therapies directed against the activation of erbB2 in breast cancer treatment.

Body

Aim1: Investigate EGF dependent erbB1 homo-oligomerization and erbB1-erbB2 hetero-oligomerization in vivo and identify structural determinants of hetero-oligomerization.

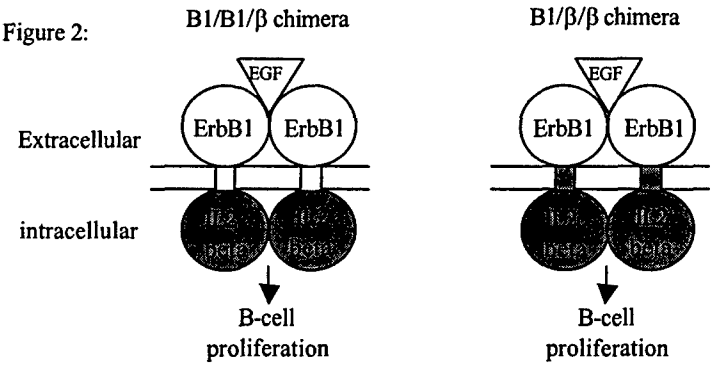
As described in the previous annual report (July 2002), I had constructed the various erbB1/IL2 and erb**B2**/IL2 receptor chimerae expression plasmids (Aim 1a and Aim3a) and created B-cell lines stably expressing these receptor chimerae (Aim 1b). Although generation of stable cell lines expressing only one chimera was feasible, creation of stable cell lines expressing two receptor chimerae was difficult to achieve. As a consequence, I was not able to generate a cell line expressing both erbB1/IL2 receptor chimerae (as shown in figure 1) to demonstrate epidermal growth factor (EGF) dependent erbB1 homo-oligmerization (as a positive control for this system). In figure 1, the heteromeric interaction of the intracellular beta and gamma chain of the IL2 receptor mediates B or T-cell proliferation in the absence of IL2.

Figure 1:



Despite this problem, I was able to alternatively show EGF dependent erbB1 homo-oligomerization through use of Baf3 (mouse pro-B cell) cell-lines stably expressing only a single erbB1/IL2R beta chimera (Aim 1c); it has been shown that in this cell line only, a homomeric interaction of the intracellular beta chain of the IL2 receptor can also mediate a proliferative signal (figure 2).

Figure 2:



In following, I previously reported (July2002) that treatment of Baf3 cells expressing either chimerae depicted in figure 2 with EGF resulted in IL2 independent proliferation (although not as robust as expected), as measured by cellular incorporation of tritium labeled thymidine (figure 3). Chimera expression level at the cell surface was determined by flow cytometry, using a PE conjugated antibody directed to the extracellular region of erbB1 (figure 4).

Figure 3:

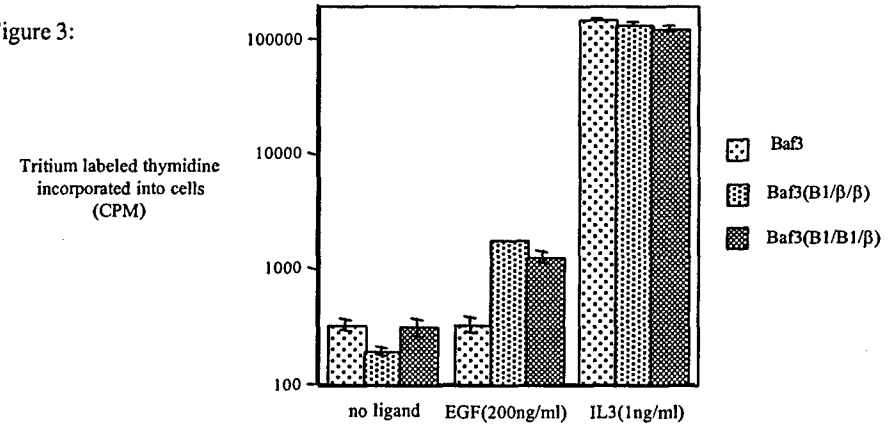
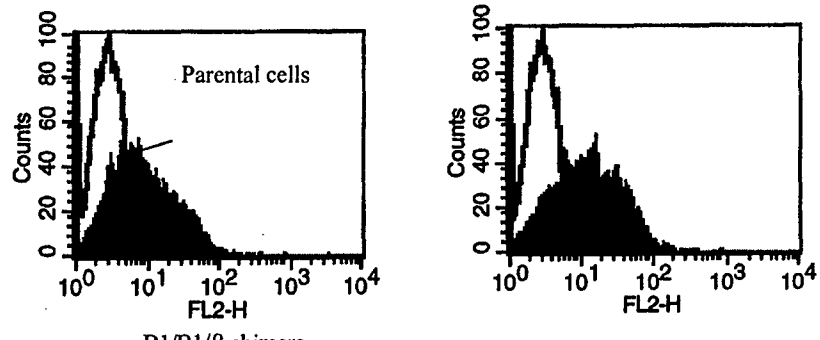


Figure 4:

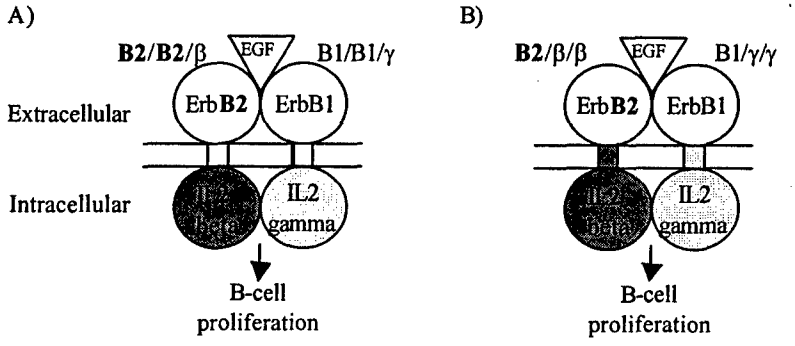


B1/B1/β chimera expression in Baf3

B1/β/β chimera expression in Baf3 cells

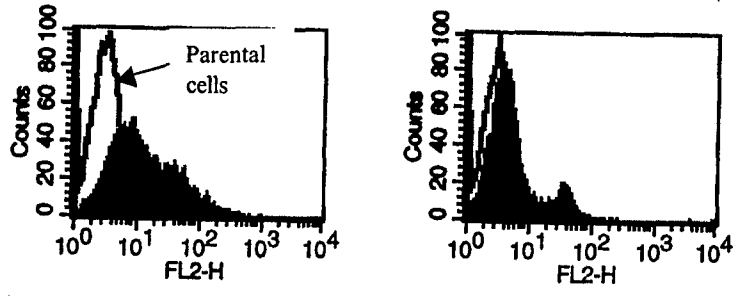
As mentioned earlier, expression of two different chimeric receptors in the same population of cells proved to be quite difficult; however I nevertheless was able to test erbB1 and erbB2 hetero-oligomerization (Aim1d) as depicted in figure 5. EGF treatment of Baf3 cells expressing both erbB1 and erbB2 receptor chimerae (as shown in figure 5) should result in hetero-oligomerization of the extracellular domains of erbB1 and erbB2. This in turn will result in the intracellular association of the IL2R beta and gamma chains mediating B-cell proliferation. In Baf3 cells expressing only an erbB1/IL2R gamma chimera, EGF will enable homo-oligomerization of the erbB1 chimerae, but the intracellular IL2R gamma-gamma association will not yield a proliferative response. In Baf3 cells expressing only an erb**B2**/IL2R beta chimera, EGF will not bind to nor enable homo-oligomerization of the erbB2 chimera. Thus a proliferative signal should not be seen in this case as well.

Figure 5:



As previously reported, I generated stable cell lines expressing both erb**B2** and erbB1 receptor chimerae by stably transfecting in the erbB2 chimera, selecting for expressing cells by fluorescence activated cell sorting (via an erbB2 antibody directed against the extracellular domain), and subsequently stably transfecting in the erbB1 chimera (Aim 1b). I was able to generate cell lines expressing only one receptor chimerae somewhat easily, however upon introduction of the second chimera, I was only able to observe expression of this receptor only in a small percentage of the cells even after selection of expressing cells by fluorescence activated cell sorting (figure 6). Similar expression profiles were observed in cells expressing both the **B2**/β/β and B1/γ/γ receptor chimerae.

Figure 6:



With these cells expressing both chimeric receptors (despite the expression problem of the 2nd stably transfected receptor- the B1/B1/γ or B1/γ/γ chimera), I was able to demonstrate EGF dependent hetero-oligomerization between erbB1 and erbB2 (as previously reported)(Aim1d). In the case of figure 5A, EGF treatment of cells expressing both chimerae yielded a proliferative response (see figure 7A). Cells expressing the erbB2/IL2R beta chimera alone yielded no response when treated with EGF (figure 7A), as expected for

reasons mentioned above. Cells expressing the erbB1/IL2R gamma chimera alone have been generated but have not been tested yet in this assay; however we predict that upon EGF treatment, no proliferative effect would be seen as mentioned above. Thus EGF dependent hetero-oligomerization can be observed between the erb**B2/B2**/β and erbB1/B1/γ chimerae. Similar results were observed in the case for figure 5B (see figure 7B), although a more robust proliferative signal was observed in this case. Given these results, we therefore have previously established that this *in vivo* system will be useful in dissecting the mechanism of hetero-oligomerization between erbB1 and erbB2. Furthermore, the results in figure 7B (also see figure 5B) suggest that the extracellular domains of erbB1 and erbB2 are sufficient for EGF dependent hetero-oligomerization *in vivo* (Aim 1d). It should be recognized that the results addressing structural requirements for erbB1-erbB2 hetero-oligomerization are in the context of receptor overexpression and ligand saturation. It is not clear whether other domains are required in the context of physiological receptor expression levels and physiological levels of circulating ligand.

Figure 7A:

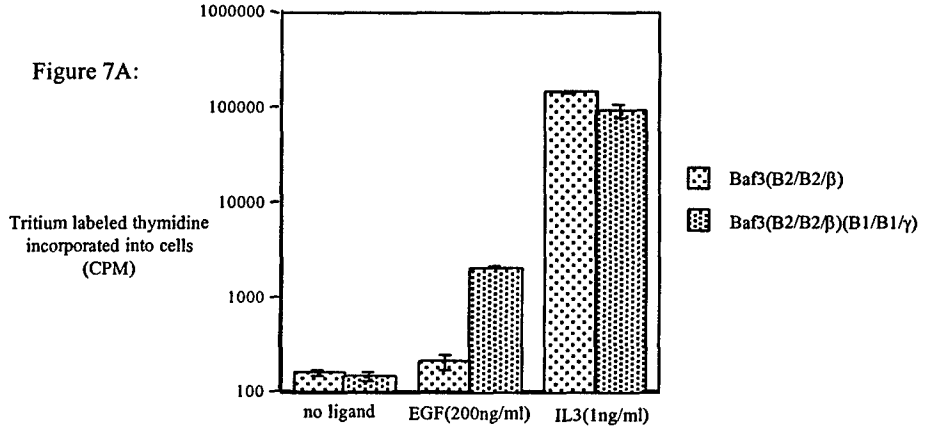
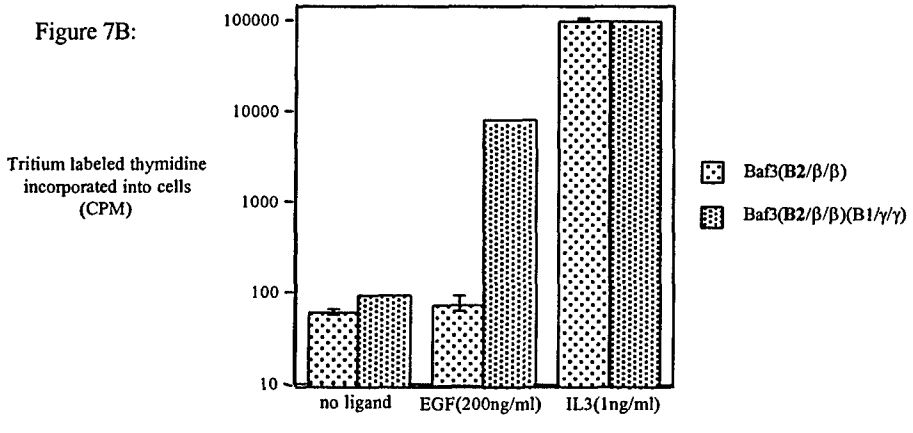


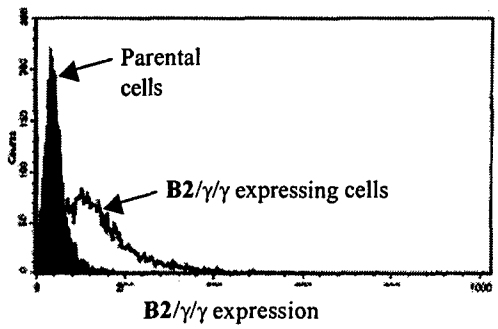
Figure 7B:



The results presented in the annual summary for July 2002 basically answer most of the questions addressed in aim 1. Efforts described in the annual summary for July 2003 were focused in generating stable cell lines which simultaneously express two or more receptor chimerae sufficiently, as improvement of the proliferative signal observed for erbB receptor hetero-oligomerization should enable us to address aim 1e, aim 2, and aim 3 in a more efficient manner. We have tried various techniques to resolve this issue, such as isolation of expressing cells by limiting dilution or by fluorescence activated cell sorting (using a PE-conjugated antibody directed against the extracellular region of either erbB1 or erbB2) and have achieved

limited success. We therefore chose to use retroviruses as a means of efficiently generating cell lines which express two or more chimeric receptors of interest. As a first step, we generated chimeric receptor constructs outlined in Aim 1a in the appropriate vector background required for virus production (repeat Aim1a and Aim3a using a different vector backbone). We subsequently tested expression of the various receptor chimera of virus infected cells by flow cytometry (using a PE-conjugated antibody directed against the extracellular region of either erbB1 or erbB2) and found that the erbB1 or erbB2 chimera are expressed in a significant fraction of cells harvested right after drug selection. A representative expression profile is shown in figure 8.

Figure 8:



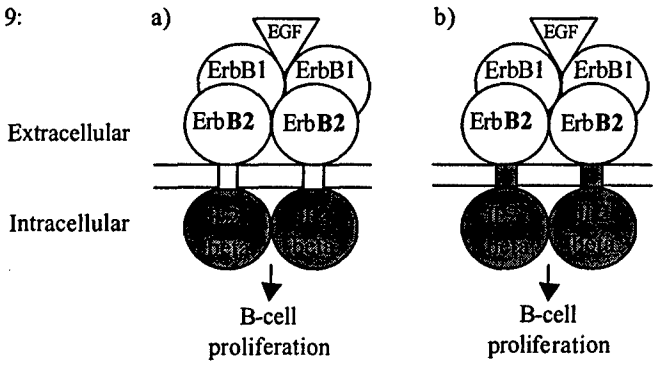
As seen in figure 8, the majority of cells express chimeric receptor immediately after virus infection and drug selection. In contrast, cells lines generated by stable transfection express chimeric receptor in only a small percentage of cells (method used in the previous annual summary). These require an additional selection step of sorting by flow cytometry (selection is via PE- conjugated antibody directed against the extracellular domain of erbB1 or erbB2) to enrich the population for expression of only one chimeric receptor. Introduction of a second chimeric receptor by similar means yields a very poor expression profile even after enrichment by cell sorting. Thus based on figure 8, we predicted that use of retrovirus is more efficient in generating single chimeric receptor expressing cell lines and is likely to also be more efficient in generating cell lines expressing two or more chimeric receptors.

Since the 2003 report, we have recently experienced much technical difficulty in generating virus that can even express the (singly) introduced chimeric receptor robustly despite the initial results shown in figure 8. As a consequence, we have and currently are devoting much time in obtaining new reagents (such as the cell lines that produce virus of interest) and repeating the process of generating cell lines stably expressing chimeric receptors described above. We have also currently constructed bicistronic expression plasmids (that possess two chimeric receptors of interest) and plan to stably transfect these in the appropriate cells as an alternate means to efficiently generate usable cell lines needed to address the subsequent untested aims.

Aim3: Determine if erbB1 and erbB2 interact as a hetero-tetramer rather than a hetero-dimer.

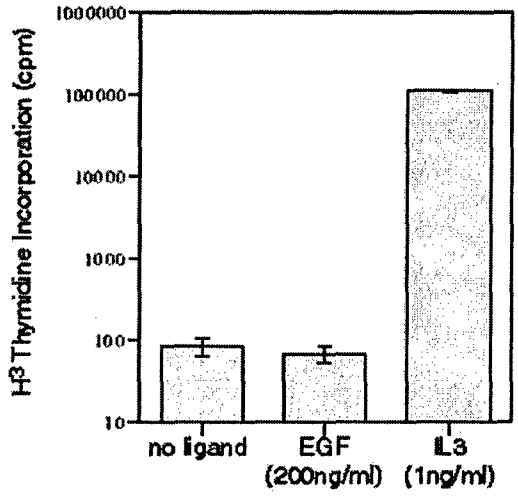
As described in the 2003 report, we planned to address this aim by determining if EGF can mediate homo-oligomerization of erbB2 in an erbB1 dependent manner (see figure 9). Thus, if this model is correct, EGF treatment of cells expressing an erbB2/IL2 beta chimera and erbB1 (full length or a truncation lacking the intracellular portion) should result in erbB2/IL2 beta chimera homo-oligomerizaton and subsequent IL2 independent B-cell proliferation. As previously described, homomeric interaction of the intracellular beta chain of the IL2 receptor interaction can mediate B-cell proliferation only in Baf3 cells.

Figure 9:



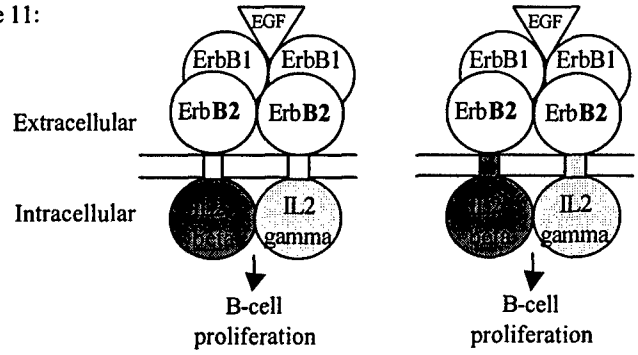
We were able to make an initial attempt to address Aim 3 using standard methods of stable transfection to generate the double stable illustrated in figure 9 (Aim 3c). Despite the problem with introduction of a second chimeric receptor into these cells, introduction of a truncated erbB1 construct (possessing only the extracellular domain, the transmembrane domain, and only a myc tag in the intracellular portion), into cells already expressing an erb**B2**/IL2 beta chimera (**B2/B2**/β or **B2**/β/β) proved to be efficient and robust in expression. As shown in figure 10, treatment of cells expressing receptors depicted in figure 9a (truncated erbB1 and erbB2/B2/β) with EGF yielded no proliferative response. This suggests that erbB1 and erbB2 do not interact as a hetero-tetramer. Although, as a positive control, treatment of these cells with an erbB2 antibody known to cross-link the extracellular domain also did not yield a proliferative response. Given that the positive control experiment did not yield the appropriate response, the results outlined in figure 10 can not be interpreted with confidence; it is possible that the chimera generated does not allow the intracellular beta chain to interact in the proper geometry to promote proper signaling towards proliferation. However, we have previously observed that this chimera (**B2/B2**/β or **B2**/β/β) is fully functional in promoting a proliferative signal during EGF dependent hetero-oligomerization with an erbB1/IL2 gamma chimera (see figure 5 and 7).

Figure 10:



Therefore, to address this aim in an alternate manner, we plan to assess EGF and erbB1 dependent erbB2 homo-oligomerization in the context of an intracellular beta chain and gamma chain association as originally proposed (figure 11). Since we have observed that homomeric interaction of IL2 receptor intracellular beta chain yields only a slight to modest proliferative response (figure 3), as compared to heteromeric interation of the intracellular beta and gamma chains (figure 7), it is possible that the heteromeric interaction of the intracellular beta and gamma chains of the IL2 receptors may be less sensitive to proper geometry constrictions as a function of the chimeric receptors. We are therefore currently working on generating cell lines which stably express all three receptors (as illustrated in figure 11) by retrovirus (or other methods) and plan to retest this aim accordingly.

Figure 11:



Other work accomplished for the July 2004 summary outside of original research proposal

Since we were having significant problems generating cell lines stably expressing two or more chimeric receptors, it was not clear if we would be able to develop a robust system to address the mechanism of erbB1 and erbB2 hetero-oligomerization outlined in the initial proposal. We therefore also started work on a genome-wide functional analysis of pleckstrin homology (PH) domains in the yeast *S. cerevisiae*. PH domains are well recognized as modules which target their host proteins to specific subcellular membrane locations through high affinity and stereo-specific recognition of membrane embedded phosphoinositides. This is domain is of particular interest because it is an extremely common domain (ranked as the 11th most common domain in the human genome) and is present in proteins of diverse function, particularly in many key signaling molecules that are implicated in breast cancer. Prior to our analysis, it was known that only a small fraction of PH domains studied can specifically bind particular phosphoinositides with high affinity and as a consequence target to membrane regions where this lipid is enriched, as described above. By contrast, the great majority of those studied bind phosphoinositides very weakly and with no specificity and in many instances these domains are unable to function as independent membrane targeting modules. Since these studies represent only a small fraction of known PH domains, it is possible that PH domains can specifically recognize other phosphoinositides that possess very few or no known protein binding partners. Furthermore, PH domains may commonly function as membrane targeting modules using multiple and distinct ligands (in addition to or exclusive of phosphoinositides) or the vast majority may not function in membrane targeting at all. Thus, we investigated properties for which PH domains are well known for (phosphoinositide binding and membrane targeting) in all PH domains in yeast (there are 33 as determined by the SMART database).

Surprisingly we found that only one of 33 yeast PH domains specifically recognized a particular phosphoinositide with high affinity. The great majority bound phosphoinositides promiscuously and with very low affinity, although a few in this group did bind strongly. In addition to the one PH domain (from Num1p) shown to specifically bind PtdIns(4,5)P_2 with high affinity, several other PH domains from the latter category (non-selective and low affinity phosphoinositide recognition) were targeted to specific membrane locations.

Further analysis indicated that phosphoinositide binding is important for membrane targeting in just about all cases, but other targets are most likely required given the lack of lipid binding specificity and affinity (with the exception of the PH domain from Num1p). In one case, phosphoinositide binding appears to play no role in its membrane targeting (that from the C-terminal PH domain of Opy1p). The remaining 70% of yeast PH domains are not membrane targeted at all in isolation. Thus high affinity and stereo-specific recognition of phosphoinositides and in vivo membrane targeting, properties for which PH domains are well known for, are not common in yeast. We believe these data provide an initial step in understanding the function of this common domain in proteins with diverse functions. These data were published March of 2004 in *Molecular Cell.*

Key research accomplishments

Previous (July 2002)
- Created receptor chimerae constructs of various deletions of erbB1 and erbB2 (Aim 1a)
- Created stable cell lines expressing the various receptor chimerae (Aim 1b)
  -incomplete; experienced problems in generating cell lines expressing two or more chimeric receptors
- Observed EGF dependent erbB1 homo-oligomerization using this *in vivo* reporter system (Aim1c)
- Observed EGF dependent erbB1-erbB2 hetero-oligomerization using this *in vivo* reporter system (Aim1d)
- Identified structural determinants required for EGF dependent erbB1-erbB2 hetero-oligomerization (Aim 1d)

Previous (July 2003)
- Re-made receptor chimerae constructs described in Aim1a and 3a in appropriate vector backbone to required for retrovirus production
- Re-made stable cell lines expressing only single receptor chimera using retroviruses (Aim 1b)
  -We are currently in the process of generating cells lines expressing two or more chimeric receptors using this method.
- Tested hetero-tetramer model of interaction between erbB1 and erbB2 and observed a negative result (aim 3c)
  -the result obtained for this task is not clear as control experiments did not yield the appropriate response
  -this aim will be restested in the context of figure 11 rather than figure 9 after appropriate cells lines have been generated

Current (July 2004)
- Re-made stable cell lines expressing only singer receptor chimera using retroviruses
  -this was accomplished in 2003, but was repeated due to expression problems in those previously generated cells
- Re-made receptor chimerae constructs described in Aim1a and in biscistronic vectors to allow simultaneous expression of both receptors upon a single transfection (stable) event.
- Unrelated to this proposal, we have also performed a genome-wide analysis of Pleckstrin homology domain function in budding yeast, which was published this year.

Reportable outcomes

None

Conclusions

Prior results have indicated that the *in vivo* reporter system proposed will be useful in dissecting the mechanism of hetero-oligomerization between erbB1 and erbB2. As described in my report for 2002, I observed EGF dependent erbB1 homo-oligomerization as a control for this system and I have also observed EGF dependent erbB1-erbB2 hetero-oligomerization and addressed the structural requirements for this hetero-oligomerization. At the conclusion of my 2002 report, I deemed that further work is required in establishing cell lines which express more than one receptor chimera at a sufficient level. Since then I spent considerable time using retrovirus to generate required cell lines to efficiently address the remaining tasks. In 2003, I reported that we have generated the chimeric receptor constructs in the appropriate vector backbone and have begun to generate cell lines expressing these chimerae stably. In addition, I have made an initial attempt to address aim 3 without the use of retroviruses, where the results suggest that erbB1 and erbB2 do not interact as a hetero-tetramer in an EGF dependent manner; however, the results obtained are not clear. Recently, I have experienced problems in generating cell lines stably expressing two or more chimeric receptors and spent considerable effort in troubleshooting this approach. As a consequence I was not able to make significant progress in this proposal; however, during this time I also worked on a genome-wide analysis of Pleckstin homology domain function in yeast which has been recently published.

References

none

Appendices

Reprint manuscript attached next page

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Genome-Wide Analysis of Membrane Targeting by *S. cerevisiae* Pleckstrin Homology Domains

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Summary

Pleckstrin homology (PH) domains are small protein modules known for their ability to bind phosphoinositides and to drive membrane recruitment of their host proteins. We investigated phosphoinositide binding (in vitro and in vivo) and subcellular localization, and we modeled the electrostatic properties for all 33 PH domains encoded in the *S. cerevisiae* genome. Only one PH domain (from Num1p) binds phosphoinositides with high affinity and specificity. Six bind phosphoinositides with moderate affinity and little specificity and are membrane targeted in a phosphoinositide-dependent manner. Although all of the remaining 26 yeast PH domains bind phosphoinositides very weakly or not at all, three were nonetheless efficiently membrane targeted. Our proteome-wide analysis argues that membrane targeting is important for only ~30% of yeast PH domains and is defined by binding to both phosphoinositides and other targets. These findings have significant implications for understanding the function of proteins that contain this common domain.

Introduction

Pleckstrin homology (PH) domains are common modules of ~120 aa found in proteins involved in signaling, cytoskeletal organization, membrane transport, and modification of phospholipids. The core PH domain fold consists of a seven-stranded β sandwich capped off by a characteristic C-terminal α-helix and is also seen in several domain classes, including phosphotyrosine binding (PTB) domains, Ena/Vasp homology (EVH-1) domains, and a Ran binding domain (Blomberg et al., 1999; Lemmon and Ferguson, 2000). The PH domain fold appears to represent a structural module adaptable to several different binding functions, interacting with phosphoinositides in some cases and with protein targets in others (Lemmon and Ferguson, 2000).

The PH domain is the 11th most common domain in humans, with ~252 examples (International Human Genome Consortium, 2001). Despite this prevalence and the fact that 14 PH domain structures have been determined, the majority of PH domains are poorly understood, and it is not known how their functions vary across the genome. PH domains are best known for their ability to bind phosphoinositides and to be targeted to cellular membranes. For example, the PH domain from phospholipase C-δ_1 (PLCδ-PH) binds with high affinity and specificity to PtdIns(4,5)P_2 and is now frequently used as a probe to localize this phosphoinositide in living cells (Balla et al., 2000). Several PH domains (e.g., from Grp1 and PKB) bind tightly and specifically to the products of agonist-dependent phosphoinositide 3-kinases and drive signal-dependent recruitment of their host proteins to the plasma membrane.

PH domains with specific phosphoinositide recognition properties have been well studied and can be identified based on a sequence motif in the β1/β2 loop between the first two strands of the β sandwich (Dowler et al., 2000; Isakoff et al., 1998). However, more than 80% of PH domains do not have this β1/β2 loop sequence motif (or related sequences), and many have been shown to bind phosphoinositides only weakly and with little specificity (Kavran et al., 1998; Rameh et al., 1997; Takeuchi et al., 1997). Since only a small fraction of PH domains has been analyzed, it remains possible that there are examples capable of specifically recognizing phosphoinositides in a structurally distinct way. Alternatively, PH domains that do not bind phosphoinositides may be membrane targeted by binding to other ligands. Some PH domains may not be membrane targeted at all.

To investigate these possibilities from a genomic perspective, we analyzed all 33 *S. cerevisiae* PH domains identified (in late 2001) by the SMART database (Schultz et al., 2000). We investigated phosphoinositide binding in vitro and in vivo, as well as membrane targeting by the isolated PH domains. We also analyzed the electrostatic properties of structural models of each PH domain. Our results provide a genome-wide view of PH domain function, suggest that several PH domains have more than one binding target in cellular membranes, and argue that the best-known characteristics of PH domains are in fact the least common.

Results and Discussion

In Vitro Phosphoinositide Binding Specificity

We first investigated the phosphoinositide binding specificity of each yeast PH domain, employing a semiquantitative lipid overlay approach that has been used extensively in initial characterization of membrane targeting domains (Dowler et al., 2000; Kavran et al., 1998). Using domain boundaries guided by earlier structural studies of PH domains, we could generate sufficient protein for

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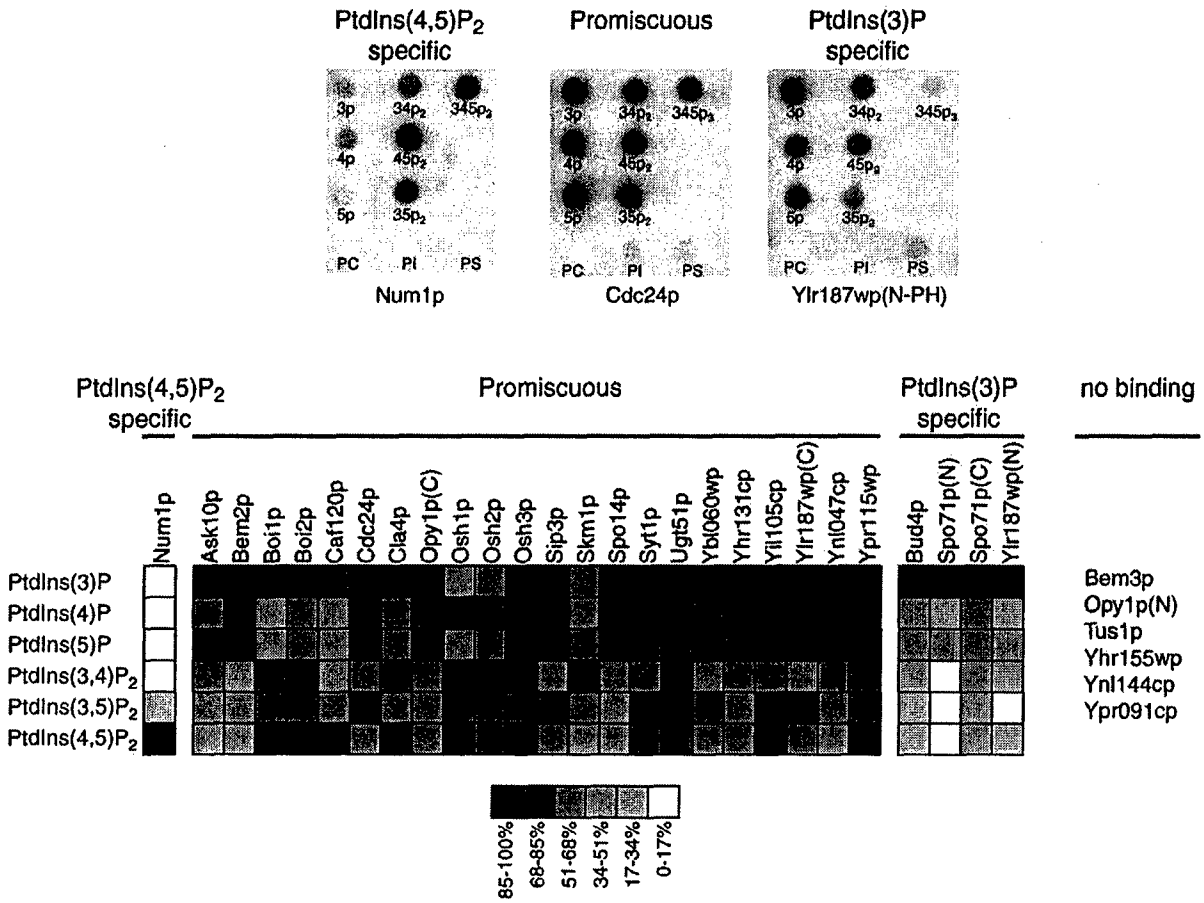

Figure 1. Phosphoinositide Binding Specificity of Yeast PH Domains Using a Lipid Overlay Method

In the upper panel, a representative lipid overlay experiment is shown for each specificity group (except the "no binding" group). In the lower panel, a semiquantitative representation of lipid overlay results for each PH domain is shown. The phosphoinositide that bound most strongly to each PH domain was arbitrarily scored as 100%. For a representative experiment, binding to other phosphoinositides is expressed as a percentage of this value and the boxes colored according to the legend. This scoring scheme does not allow comparison of affinities between PH domains, but color variation gives an impression of specificity.

this analysis for all 33 yeast PH domains (from 30 different proteins). Each PH domain was expressed as a GST fusion, labeled with ^{32}P, and used to probe nitrocellulose membranes bearing spots of relevant phosphoinositides. We anticipated that this analysis would identify *S. cerevisiae* PH domains with specific phosphoinositide targets and/or with novel specificities. Contrary to these expectations, the overwhelming impression was instead one of nonspecific, or promiscuous, phosphoinositide binding by PH domains (Figure 1). Some 67% of yeast PH domains (22 of 33) bound all phosphoinositides tested in this assay, with no clearly preferred binding partner, and six PH domains showed no detectable phosphoinositide binding. One PH domain (from Num1p) specifically recognized PtdIns(4,5)P_2 and four showed a preference for PtdIns(3)P, as reported for certain human and *Arabidopsis* PH domains (Dowler et al., 2000). In summary, our lipid overlay studies argue that specific phosphoinositide binding is a property of very few (<15%) yeast PH domains.

Only Seven Yeast PH Domains Bind Strongly to Phosphoinositides

Lipid overlay studies give an impression of phosphoinositide binding specificity but provide no useful information about binding affinities. To assess the strength of phospholipid binding by each yeast PH domain and to reassess specificity in a more physiological setting, we used surface plasmon resonance (SPR). Each GST/PH domain fusion (at ≥3 μM) was tested for binding to membranes containing phosphoinositides at 3% (mole/mole) in a phosphatidylcholine (DOPC) background (Figure 2). This was done for 27 PH domains (six failed to produce sufficiently well), using membranes with 3% PtdIns(4,5)P_2, PtdIns(3,5)P_2, PtdIns(3)P, or PtdIns(4)P. Binding to PtdIns(4,5)P_2 could be detected for just seven yeast PH domains (Figure 2A): those from Num1p, Boi1p, Boi2p, Cla4p, Osh1p, Osh2p, and Skm1p. In all other cases, SPR signals of less than 400 response units (RUs) were obtained, which are negligible compared with the 2000–4000 RUs measured for the seven strongly binding

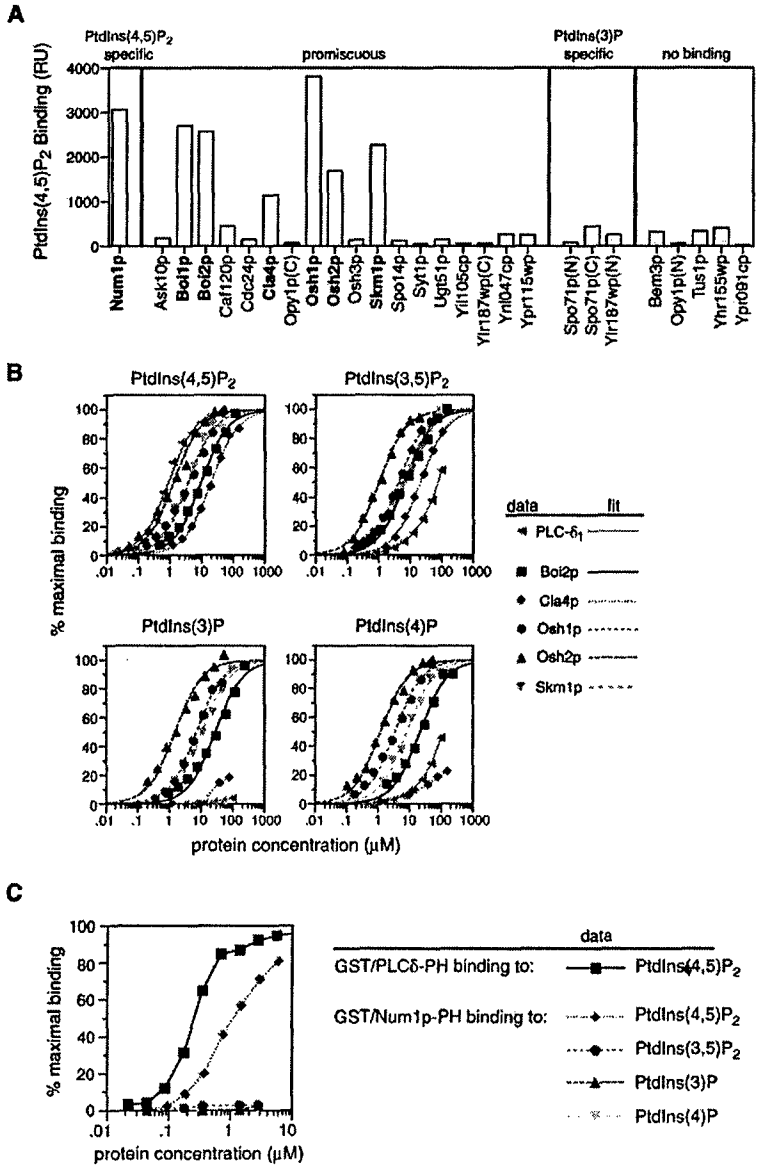

Figure 2. Surface Plasmon Resonance Analysis of Phosphoinositide Binding by Yeast PH Domains

(A) Each GST/PH fusion was flowed at ≥3 μM over a sensorchip containing 3% (mole/mole) PtdIns(4,5)P_2 in DOPC. PH domains that gave signals >1000 RUs are listed in bold type and were further analyzed. The PH domains from Bem2p, Bud4p, Sip3p, Ybl060wp, Yhr131cp, and Ynl144cp could not be produced in sufficient quantities for analysis with this approach.

(B) PH domains that bound significantly in (A) were subjected to more detailed binding analysis. Monomeric PH domains were flowed at a series of concentrations across surfaces containing 3% (mole/mole) PtdIns(4,5)P_2, PtdIns(3,5)P_2, PtdIns(3)P, or PtdIns(4)P. Binding signals are plotted against protein concentration, and the best-fit curves (with K_D values reported in Table 1) are superimposed. Data for PLCδ-PH are shown for comparison. Results are representative of at least three repeats, with errors given in Table 1.

(C) Phosphoinositide binding curves for the GST/Num1p-PH protein, as described in (B). Data for GST/PLCδ-PH are shown for comparison.

yeast PH domains or for a PLCδ-PH positive control. Essentially identical results were obtained with PtdIns(3,5)P_2, PtdIns(3)P, and PtdIns(4)P, although the Num1p and Cla4p PH domains (which show at least some specificity) gave weaker signals with other lipids (see below). The PH domains that appear PtdIns(3)P specific in Figure 1 did not bind detectably to this lipid in BIAcore studies, whereas positive-control PX and FYVE domains gave robust responses on the same sensorchip surfaces (data not shown).

Thus, only seven yeast PH domains bind phosphoinositides in vitro with micromolar or stronger K_D values as GST fusions. For the remainder that we tested, we estimate K_D values >20 μM, even with the avidity effect afforded by GST-mediated dimerization (Klein et al., 1998). In about half of the cases, additional experiments using 20–50 μM GST/PH fusion protein also gave no binding signal, placing K_D > 200–500 μM. Lipid overlay experiments nonetheless showed clear phosphoinositide binding for these cases, showing the sensitivity of this approach and illustrating that caution must be exercised in interpreting its results.

Most Yeast PH Domains that Bind Strongly to Phosphoinositides Show No Headgroup Specificity

We next quantitated phosphoinositide binding for the seven PH domains with positive signals in Figure 2A. To avoid well-documented avidity effects resulting from fusion to GST (Klein et al., 1998), we generated monomeric forms of each PH domain (except Num1p-PH and Boi1p-PH, which did not express well) as previously described (Ferguson et al., 1995). Figure 2B shows curves for binding of monomeric PH domains to each phosphoinositide found in yeast, with monomeric rat PLCδ-PH (Lemmon et al., 1995) as a comparative con-

Table 1. K_D Values for Phosphoinositide Binding by Monomeric Yeast PH Domains

PH Domain	PtdIns(4,5)P_2	PtdIns(3,5)P_2	PtdIns(3)P	PtdIns(4)P
Boi2p	9.7 ± 0.8 μM	6.6 μM	19.5 ± 11.7 μM	20.0 ± 1.3 μM
Cla4p	20.4 ± 6.4 μM	20.2 ± 0.4 μM	>100 μM	>100 μM
Osh1p	3.0 ± 1.0 μM	3.5 ± 0.8 μM	6.2 ± 1.3 μM	2.8 ± 0.8 μM
Osh2p	1.1 ± 0.3 μM	1.0 μM	1.5 ± 0.2 μM	1.3 ± 0.2 μM
Skm1p	3.9 ± 0.4 μM	6.4 μM	8.0 ± 3.7 μM	8.2 ± 0.4 μM
Rat PLC-δ_1	0.68 ± 0.28 μM	76.0 ± 4.7 μM	>100 μM	131 ± 19 μM

Data are from BIAcore experiments as shown in Figure 2B. Errors represent the standard deviation for K_D values obtained in at least three independent experiments.

trol. Best-fit K_D values are listed in Table 1. In agreement with our dot blot analyses, the Boi2p, Osh1p, Osh2p, and Skm1p PH domains showed little to no specificity, binding similarly to all four phosphoinositides. Equivalent binding characteristics were previously reported for the PH domain from OSBP, a mammalian homolog of Osh1p and Osh2p (Levine and Munro, 2002), and for Boi1p-PH (Hallett et al., 2002). Cla4p-PH showed a distinct preference for bisphosphorylated over monophosphorylated lipids, binding PtdIns(4,5)P_2 and PtdIns(3,5)P_2 with K_D values of ~20 μM but significantly less well to PtdIns(3)P and PtdIns(4)P (K_D > 100 μM). Parallel control studies using the same membrane surfaces confirmed the >100-fold preference of PLCδ-PH for PtdIns(4,5)P_2 over other phosphoinositides.

Num1p-PH Is the Only Yeast PH Domain that Binds Phosphoinositides Specifically and Strongly

Num1p-PH is the only PH domain that showed specificity in overlay studies (Figure 1) and measurable binding in Figure 2A. To analyze phosphoinositide binding by Num1p-PH in more detail, we used the highly expressed GST/Num1p-PH fusion because we could not produce large quantities of monomeric Num1p-PH. GST/Num1p-PH bound PtdIns(4,5)P_2 with an apparent K_D of ~1 μM, compared with ~0.25 μM measured for GST/PLCδ-PH (Figure 2C). By contrast with the five yeast PH domains analyzed in Figure 2B, Num1p-PH did not bind detectably to PtdIns(3,5)P_2, PtdIns(3)P, or PtdIns(4)P. Thus, Num1p-PH is a high-affinity PtdIns(4,5)P_2-specific PH domain that resembles PLCδ-PH in its phosphoinositide recognition properties. Our data suggest that Num1p-PH is the *only* PH domain in *S. cerevisiae* capable of specific and high-affinity phosphoinositide binding.

Only Six Yeast PH Domains Are Strongly Plasma Membrane Targeted, and Just Three of These Exhibit High-Affinity Phosphoinositide Binding

We were next interested to correlate in vitro phosphoinositide binding properties with subcellular localization of isolated yeast PH domains. With PH domains covering a wide range of phosphoinositide binding strengths (Table 1), we anticipated that this should provide insight into the affinity requirements for phosphoinositide-dependent membrane targeting in vivo, particularly given concerns that phosphoinositides may not be the only cellular targets of PH domains (Balla et al., 2000; Levine and Munro, 2002). We generated enhanced green fluorescent protein (EGFP) fusions of each isolated *S. cerevisiae* PH domain and investigated bulk localization in HeLa and yeast cells. Of the 33 EGFP/PH fusions, 21 showed only featureless and diffuse localization in HeLa and yeast cells. This group included all six PH domains that did not bind phosphoinositides in lipid overlay experiments. Six PH domains showed some nuclear localization, the relevance of which we have not yet established.

Of most interest are the six PH domains that show significant plasma membrane (PM) localization in both yeast and HeLa cells (from Cla4p, Num1p, Opy1p-C, Skm1p, Yil105cp, and Ynl047cp; Figure 3), several appearing to be enriched in patches at the PM (e.g., Yil105cp-PH and Opy1p C-PH). In addition, the Osh1p and Osh2p PH domains are localized to intracellular structures (Figure 3), identified as Golgi in previous studies of these two PH domains (Levine and Munro, 2001). Both of the Golgi-localized PH domains bind phosphoinositides strongly in vitro. However, this was true for only three of the six PM-localized PH domains (Num1p-PH, Cla4p-PH, and Skm1p-PH). The remaining three (Opy1p C-PH, Yil105cp-PH, and Ynl047cp-PH) bind phosphoinositides too weakly to be detectable using SPR, yet their PM fluorescence in yeast cells is the most intense (and is stronger than for PLCδ-PH). Thus, high-affinity phosphoinositide binding in vitro is not required for in vivo membrane targeting, suggesting that there are additional (or alternative) binding targets in the membrane.

Since analysis of EGFP fusion proteins will miss membrane recruitment mediated by low-abundance targets, we also used a more sensitive Ras recruitment assay (Isakoff et al., 1998). PH domains fused to a constitutively active Ha-Ras mutant can target active Ras to the membrane and thus rescue growth of *cdc25^{ts}* yeast. As shown in Figure 4A, PLCδ-PH rescues *cdc25^{ts}* yeast growth at 37°C by targeting the Ras fusion to PtdIns(4,5)P_2 in the yeast PM, whereas the dynamin PH domain does not. All six PH domains that were PM targeted as EGFP/PH fusions could recruit Ras to the membrane efficiently in this assay (Figure 4A). The Golgi-localized Osh1p and Osh2p PH domains also drove robust Ras recruitment, suggesting either partial PM localization (as in HeLa cells; Figure 3) or the ability of activated Ras to signal from internal membranes (Chiu et al., 2002). Of 11 additional PH domains analyzed with this approach (Figure 4A), 7 could not drive membrane recruitment of Ras, consistent with their diffuse cytoplasmic localization as EGFP/PH fusions. Boi2p-PH, which was not membrane targeted as an EGFP fusion protein [but binds PtdIns(4,5)P_2 with K_D ~10 μM], did promote membrane recruitment of Ras, as did Caf120p-PH (albeit weakly).

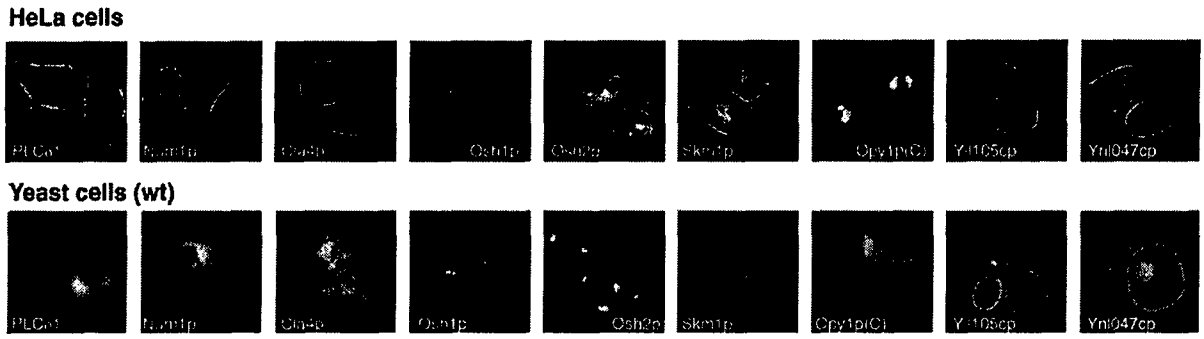

Figure 3. Subcellular Targeting of EGFP-Fused Yeast PH Domains in HeLa and Yeast Cells

Fluorescence micrographs are shown only for cases in which significant targeting was observed. Results for all other PH domains in yeast are given in Table 2.

Ask10p-PH and Ybl060wp-PH, which were both seen in the nucleus as EGFP fusions, could also target Ras to yeast membranes. Ras rescue studies thus confirmed all of the observations made with EGFP fusions but also suggested that many yeast PH domains (at least seven) have no significant membrane-targeting ability. The origin of the rescue seen with the Ask10p, Ybl060wp, and Caf120p PH domains is not yet clear and may result

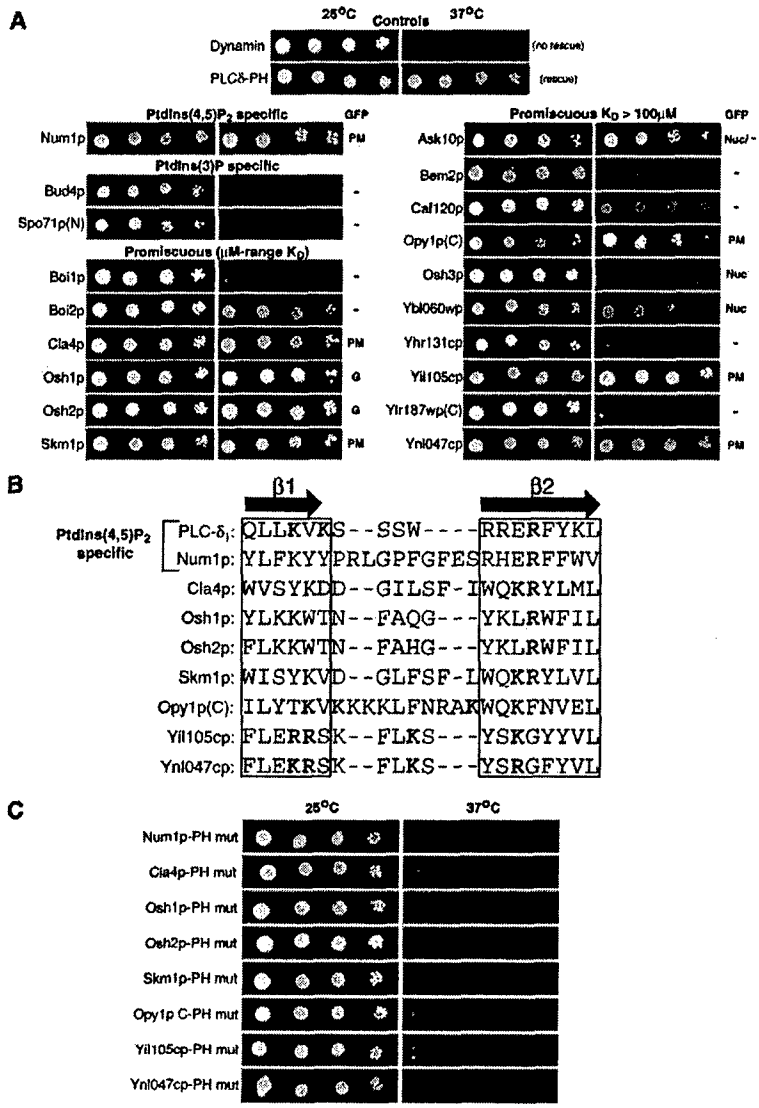

Figure 4. Membrane Targeting of Wild-Type and Mutated Yeast PH Domains Assessed by Ras Rescue

(A) Serial dilutions of *cdc25*^{ts} yeast cultures expressing the noted PH domain/RasQ61L(Δf) fusion were spotted onto duplicate selection plates lacking leucine and incubated at the permissive temperature (25°C) or restrictive temperature (37°C) for 7 to 8 days. Membrane targeting by the fused PH domain is required for yeast growth at 37°C (Isakoff et al., 1998). At the right of each experiment it is noted whether the EGFP/PH fusion was localized in yeast cells to the plasma membrane (PM), Golgi (G), nucleus (Nuc), or only the cytoplasm (–).

(B) Sequence alignment of the β1/β2 loop region of yeast PH domains that were membrane targeted as EGFP fusion proteins. The basic residues colored red [critical for PtdIns(4,5)P_2 binding in the case of PLCδ-PH] were simultaneously mutated to alanine to disrupt each predicted phosphoinositide binding site.

(C) Ras rescue experiments show that mutation of the presumed phosphoinositide binding site abolishes the ability of all eight PH domains to drive membrane recruitment. Western blot controls (data not shown) confirmed that Ras/PH fusion expression was not impaired by these mutations.

either from very weak phosphoinositide binding or from recognition of low-abundance membrane targets, both of which would be missed in EGFP fusion studies.

Membrane Targeting by PH Domains Is Impaired by Mutations in the Putative Phosphoinositide Binding Site

Every PH domain that was membrane targeted as an EGFP or Ras fusion also bound phosphoinositides in one of our assays (Table 2). We therefore asked whether mutations that abolish phosphoinositide binding also prevent membrane targeting in all cases. We focused on the eight PH domains targeted to the PM or elsewhere as EGFP fusion proteins. These are listed in Figure 4B alongside the β1/β2 loop sequences likely to constitute their phosphoinositide binding sites (Lemmon and Ferguson, 2000). For each PH domain, we replaced the basic residues, colored red in Figure 4B, with alanine, guided by the location of positively charged side chains in the PLCδ-PH β1/β2 loop that contact the PtdIns(4,5)P_2 headgroup (Ferguson et al., 1995). These mutations abolished or greatly diminished phosphoinositide binding in each case (data not shown), as assessed using SPR (for Num1p, Cla4p, Osh1p, Osh2p, and Skm1p PH domains) or lipid overlay experiments (for the Opy1p, Yil105cp, and Ynl047cp PH domains). This impaired phosphoinositide binding correlated in all cases with loss of membrane targeting by the PH domain, as assessed by the Ras rescue assay (Figure 4C). Western blotting with antibodies against an HA-tag in each fusion protein (data not shown) confirmed that expression levels were not affected by the mutations, excluding trivial misfolding explanations for the lack of Ras rescue.

Phosphoinositides Are Important for Recruitment of All Membrane-Targeted Yeast PH Domains Except Opy1p C-PH

The fact that β1/β2 loop mutations impair membrane targeting is consistent with a role for phosphoinositide binding in localizing these PH domains but does not provide direct evidence. Other ligands that bind to the same site on the PH domain could instead be responsible. To distinguish between these possibilities, we analyzed the localization of each EGFP/PH fusion in several *S. cerevisiae* mutants with different well-characterized alterations in phosphoinositide levels (Odorizzi et al., 2000). Using this approach, we showed that phosphoinositides play a direct role in membrane targeting in vivo of all but one (Opy1p C-PH) of the eight PH domains listed in Figure 4B.

We first investigated the effects of reducing PtdIns(4,5)P_2 levels, using *mss4^{ts}* cells. Mss4p is the PtdIns(4)P 5-kinase responsible for all yeast PtdIns(4,5)P_2 production (Desrivieres et al., 1998; Homma et al., 1998). In *mss4^{ts}* cells (AAY202; Stefan et al., 2002) at the permissive temperature (26°C), PtdIns(4,5)P_2 levels are ~57% of those in wild-type cells and fall ~3-fold further (to <20% of wild-type levels) at the restrictive temperature (Stefan et al., 2002). Levels of PtdIns(4)P, PtdIns(3)P, and PtdIns(3,5)P_2 are unaffected. In *mss4^{ts}* cells, membrane localization of Num1p-PH was difficult to discern even at the permissive temperature (Figure 5), probably because of reduced PtdIns(4,5)P_2 levels (this was also true for monomeric EGFP/PLCδ-PH). However, PM localization of the PH domains from Cla4p, Skm1p, Opy1p-C, Yil105cp, and Ynl047cp was quite clear in *mss4^{ts}* cells at 26°C. When PtdIns(4,5)P_2 levels were further reduced by shifting to 37°C, PM localization of all but the Opy1p C-terminal PH domain was greatly reduced. This finding argues that PtdIns(4,5)P_2 contributes significantly to membrane targeting of the Cla4p, Skm1p, Yil105cp, and Ynl047cp PH domains. By contrast, PM localization of Opy1p C-PH appears to be PtdIns(4,5)P_2 independent as does the intracellular punctate localization of the Osh1p and Osh2p PH domains. Similar results were obtained in *stt4^{ts}* cells at the restrictive temperature (data not shown), where inactivation of the Stt4p PtdIns 4-kinase reduces PM levels of both PtdIns(4)P and PtdIns(4,5)P_2 (Audhya and Emr, 2002; Audhya et al., 2000).

The type III PtdIns 4-kinase Pik1p appears to synthesize a Golgi-located PtdIns(4)P pool that is required for normal secretion (Audhya et al., 2000; Hama et al., 1999; Walch-Solimena and Novick, 1999). Reducing Golgi PtdIns(4)P levels by shifting *pik1^{ts}* cells to 37°C did not affect localization of the Num1p, Cla4p, Skm1p, Opy1p, Yil105cp, or Ynl047cp PH domains (data not shown). However, as previously reported (Levine and Munro, 2002), the punctate intracellular localization of Osh1p-PH and Osh2p-PH (both found at the Golgi) was substantially diminished. In *stt4^{ts}/pik1^{ts}* double mutants (Audhya et al., 2000), only Opy1p C-PH remained significantly localized (although its expression appeared to be toxic).

Another approach to manipulating cellular PtdIns(4)P and PtdIns(4,5)P_2 levels is to use yeast with mutations in phosphoinositide phosphatases. For example, in *sjl1*Δ cells, which lack the synaptojanin family PtdIns(4,5)P_2 5-phosphatase Sjl1p/Inp51p, PtdIns(4,5)P_2 levels are elevated by approximately 2-fold (Stefan et al., 2002; Stolz et al., 1998). As predicted from the findings in *mss4^{ts}* cells, this PtdIns(4,5)P_2 accumulation results in enhanced PM localization of the Num1p, Cla4p, Skm1p, Yil105cp, and Ynl047cp PH domains in *sjl1*Δ cells (Figure 5). Localization of Opy1p C-PH was only slightly enhanced (if at all), and Osh1p-PH and Osh2p-PH were unaffected. To investigate the effect of accumulating PtdIns(4)P at the PM, we also analyzed PH domain localization in cells lacking the Sac1p phosphatase. In *sac1*Δ cells, PM PtdIns(4)P (generated by Stt4p) accumulates to levels 20-fold higher than normal (Foti et al., 2001). PtdIns(3)P and PtdIns(3,5)P_2 levels are also slightly elevated (by 2-fold), and PtdIns(4,5)P_2 levels are depressed by around 75% (Foti et al., 2001). We found that Num1p-PH and Cla4p-PH are entirely cytoplasmic in *sac1*Δ cells, consistent with the reduced PtdIns(4,5)P_2 levels. The Skm1p, Yil105cp, and Ynl047cp PH domains all showed reduced but nonetheless discernible levels of PM localization. This is consistent with the fact that they bind similarly to PtdIns(4,5)P_2 and PtdIns(4)P in vitro or with the possibility that they have additional binding targets. The most dramatic effect of deleting *SAC1* was to promote strong PM localization of the Osh2p PH domain (Figure 5). Rather surprisingly (but consistent with a previous report of Levine and Munro, 2002), Osh1p-PH was not seen at the PM in *sac1*Δ cells. It therefore appears that, while Osh2p-PH can bind PtdIns(4)P at both the

Table 2. Summary of Phosphoinositide Binding Properties of Yeast PH Domains and Their Localization in Wild-Type Yeast Cells

	Ask10p	Bem2p	Bem3p	Boi1p	Boi2p	Bud4p	Caf120p	Cdc24p	Cla4p	Num1p	Opy1p N
PM	–	–	–	–	–	–	–	–	+/–	+	–
Diffuse cytoplasm	+	+	+	+	+	+	+	+	+	–/+	+
Punctate cytoplasm	–	–	–	–	–	–	–	–	–	–	–
Nucleus[a]	–/+	–	–	–	–	–	–	–	–	–	–
PI binding/specificity[b,c]	+/none[c]	+/none	–	+++/none	+++/none	+/PI(3)P	+/none	+/none	+++/PIP_2[e]	+++/PI(4,5)P_2	–
Intact protein[d]	cytoplasm	cytoplasm, bud neck	cytoplasm, bud neck	cytoplasm, bud, bud neck, cell periphery	cytoplasm, bud, bud neck, punctate	bud neck	cytoplasm, bud, bud neck	cytoplasm, nucleus	cytoplasm, bud	punctate composite	cytoplasm

	Opy1p C	Osh1p	Osh2p	Osh3p	Sip3p	Skm1p	Spo14p	Spo71p N	Spo71p C	Syt1p	Tus1p
PM	+	–	+/–	–	–	+	–	–	–	–	–
Diffuse cytoplasm	–/+	–	–	–	+	+/–	+	+	+	+	+
Punctate cytoplasm	–	+ (Golgi)	+	–	–	+/–	–	–	–	–	–
Nucleus[a]	+	–	–	+	–	–	–	–	–	–	–
PI binding/specificity[b,c]	+/none	+++/none	+++/none	+/none	+/none	+++/none	+/none	+/PI(3)P	+/PI(3)P	+/none	–
Intact protein[d]	cytoplasm	unscored	punctate composite	ambiguous	ambiguous	ambiguous	cytoplasm	unscored	unscored	unscored	unscored

	Ugt51p	Ybl060wp	Yhr131cp	Yhr155wp	Yil105cp	Ylr187wp N	Ylr187wpC	Ynl047cp	Ynl144cp	Ypr091cp	Ypr115wp
PM	–	–	–	–	+	–	–	+	–	–	–
Diffuse cytoplasm	+	–/+	+	+	–/+	+	+	–/+	+	+	–/+
Punctate cytoplasm	–	–	–	–	–	–	–	–	–	–	–
Nucleus[a]	–	+	–	–	–	–	–	+	–	–	+
PI binding/specificity[b,c]	+/none	+/none	+/none	–	+/none	+/PI(3)P	+/none	+/none	–	–	+/none
Intact protein[d]	cytoplasm	cytoplasm, bud neck	cytoplasm	mitochondria	punctate composite	cytoplasm, bud, bud neck, cell periphery	cytoplasm, bud, bud neck, cell periphery	unscored	unscored	ER	cytoplasm

[a] Nuclear localization of EGFP/PH fusion proteins may be an experimental artifact (Balla et al., 2000), and we have not attempted to interpret this finding.
[b] "+++" represents SPR-detectable binding; "+" represents binding detectable in lipid overlays but not SPR; "–" represents no binding by any assay.
[c] Preferred lipids are listed (none means no specificity).
[d] Results for intact proteins are taken from Huh et al. (2003).
[e] Cla4p-PH binds equally well to PtdIns(4,5)P_2 and PtdIns(3,5)P_2.

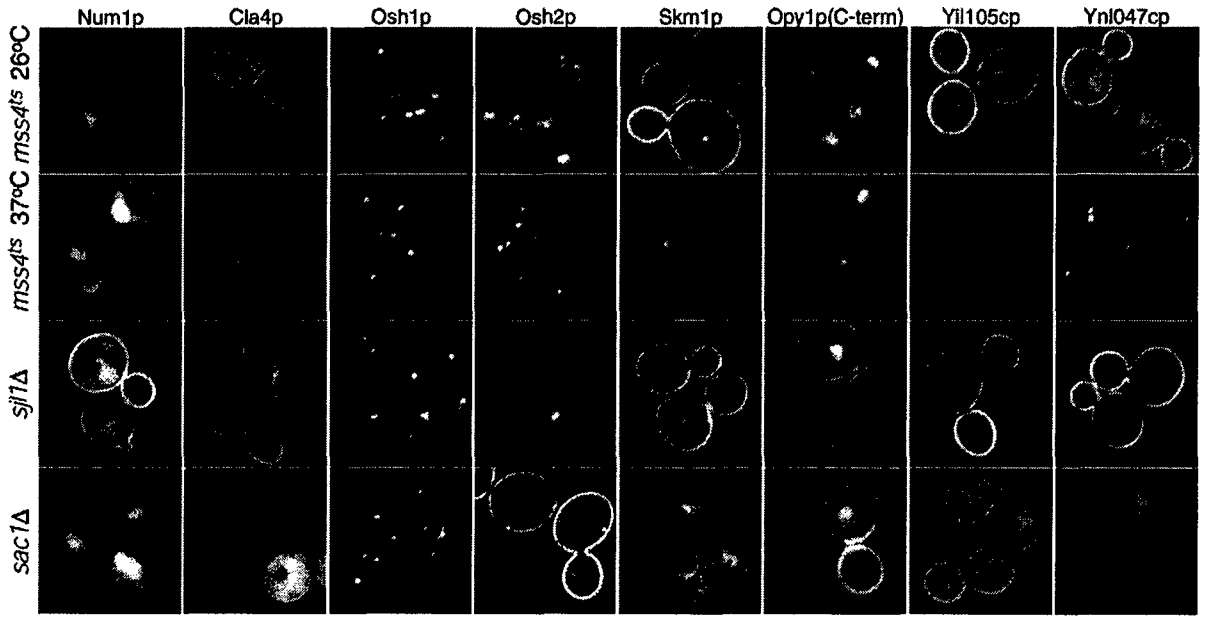

Figure 5. EGFP-PH Targeting in Yeast Cells with Altered PtdIns(4,5)P_2 or PtdIns(4)P Levels

Localization of the eight noted PH domains (fused to EGFP) was analyzed in *mss4*^{ts} cells [with reduced PtdIns(4,5)P_2 levels], grown to mid-log phase, and then incubated for 45 min at either 26°C or 37°C [where PtdIns(4,5)P_2 levels are further reduced], before being examined live by fluorescence microscopy. Localization of the same EGFP fusions in *sjl1*Δ cells [with elevated PM PtdIns(4,5)P_2] and *sac1*Δ cells [with elevated PM PtdIns(4)P] is also shown.

PM and the Golgi, Osh1p-PH recognizes PtdIns(4)P at the Golgi but not at the PM. As discussed below, this observation argues that factors in addition to phosphoinositides must influence the specificity of membrane targeting. Punctate intracellular localization was also seen in some yeast mutants for certain PH domains, such as Skm1p-PH and Ynl047cp-PH in *mss4*^{ts} cells at 37°C and Opy1p C-PH in wild-type cells (and for several cases in HeLa cells). We have not yet characterized these punctae, but the fact that they appear to differ for PH domains that have similar lipid binding specificities suggests that recruitment to them is defined by factors other than phosphoinositides.

These studies demonstrate a clear phosphoinositide dependence for PM targeting of the Num1p, Cla4p, Skm1p, Yil105cp, and Ynl047cp PH domains. For Num1p-PH and Cla4p-PH, PM localization correlates with PtdIns(4,5)P_2 levels in the membrane, consistent with their in vitro binding specificity. For the Skm1p, Yil105cp, and Ynl047cp PH domains, PM PtdIns(4)P may be able to substitute for this to some extent. Membrane localization of the Osh1p and Osh2p PH domains is also phosphoinositide dependent, and these PH domains appear to recognize distinct pools of PtdIns(4)P, in a way that cannot be explained by their phosphoinositide binding characteristics. Finally, membrane targeting of the Opy1p C-terminal PH domain is a clear exception, appearing phosphoinositide independent in these studies. As discussed in the conclusions, these findings argue that, while phosphoinositide binding clearly plays a role in many membrane targeting events observed here, in most cases additional binding targets must be invoked in the respective cellular membranes.

Sequence Characteristics of PH Domains with Different Phosphoinositide Binding and Membrane-Targeting Properties

We next asked whether the functional similarities of yeast PH domains with regards to membrane targeting and phosphoinositide binding are also reflected in their sequence relationships. The wide diversity of PH domain sequences makes this quite difficult, and no sequence patterns related to in vivo membrane targeting could be discerned. However, the genome-wide perspective showed that all PH domains with SPR-measurable phosphoinositide binding (from Num1p, Cla4p, Osh1p, Osh2p, Skm1p, Boi1p, and Boi2p) have a characteristic arrangement of basic residues in their β1/β2 loop (summarized in Figure 4B). This includes a conserved arginine in the middle of strand β2, known to be critical for PtdIns(4,5)P_2 binding by PLCδ-PH (Ferguson et al., 1995). In addition, most of the yeast PH domains that bind phosphoinositides with highest affinities (from Num1p, Osh1p, Osh2p, Boi1p, and Boi2p) have a characteristic lysine close to the end of strand β1, which also makes critical PtdIns(4,5)P_2 contacts in PLCδ-PH. Thus, all yeast PH domains with moderate to high in vitro phosphoinositide binding affinity possess a pattern of basic residues in their β1/β2 loop that resembles the well-characterized motif responsible for inositol phosphate headgroup recognition by PLCδ-PH and other mammalian PH domains (Lemmon and Ferguson, 2000). Only two other yeast PH domains have β1/β2 loop sequences that resemble this motif. One is Yhr131cp-PH, which we could not produce in sufficient quantities for SPR studies. The second is Osh3p-PH, which did not bind strongly to phosphoinositides (Figure 2A). Thus,

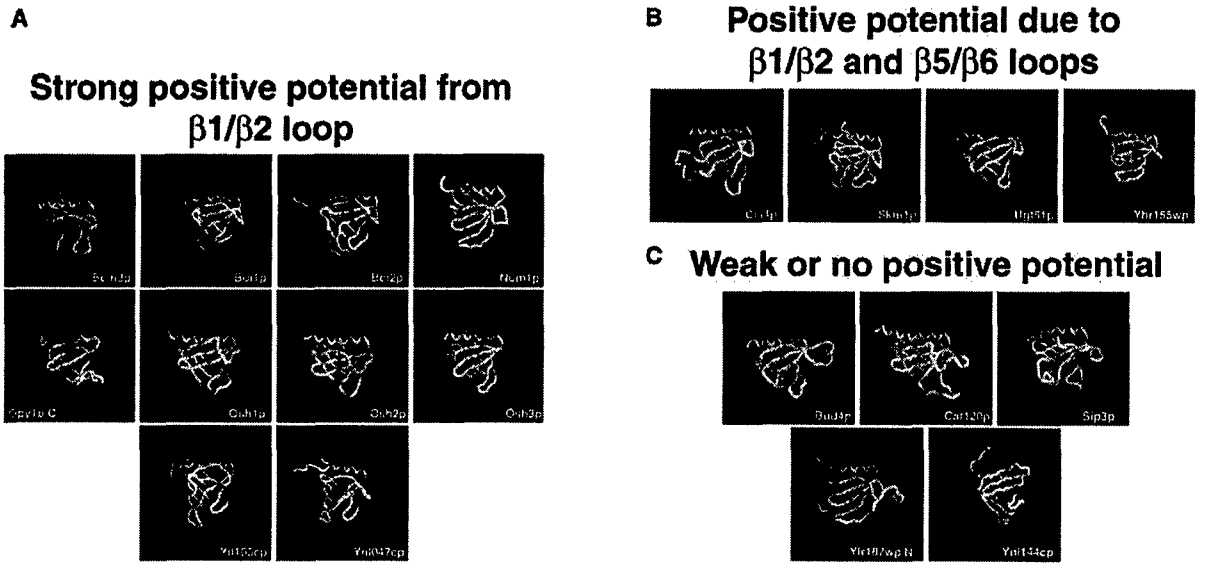

Figure 6. Electrostatic Characteristics of Modeled Yeast PH Domains

GRASP (Nicholls et al., 1991) representations are shown for the 19 PH domains for which reliable models could be obtained. Well-modeled PH domains were subdivided into three groups: those with strong positive potential arising from the β1/β2 loop (A), those with positive potential arising from the β1/β2 and β5/β6 loops (B), and those with little or no positive potential (C). Each PH domain model is shown in the same orientation, with the phosphoinositide binding side at the bottom of the panel. Alpha carbon traces are presented as white worms, and equipotential profiles are represented as blue (+25 mV) and red (−25 mV) meshes.

with Osh3p-PH as the only clear exception, the occurrence of this motif is a good predictor of moderate- to high-affinity phosphoinositide binding, and there do not appear to be alternative motifs for phosphoinositide binding (or membrane targeting) in yeast PH domains.

Molecular Modeling of PH Domains Suggests that Delocalized Electrostatic Interactions Play a Key Role in Membrane Targeting

Independently of our phosphoinositide binding and membrane-targeting studies, we also generated homology models of the three-dimensional structures of all *S. cerevisiae* PH domains, using approaches described previously (Singh and Murray, 2003). We could model 19 of the 33 PH domains well, but large insertions in the presumed loop regions prevented reliable modeling of the remaining 14. The 19 well-modeled cases include all yeast PH domains for which membrane targeting was observed and all 7 for which phosphoinositide binding was detectable with SPR. The calculated electrostatic potentials of the 19 reliably modeled PH domains, depicted as Cα worms, are illustrated in Figure 6; blue and red meshes represent positive and negative electrostatic potential, respectively. All models are shown in a similar orientation, with the predicted phosphoinositide binding site (including the β1/β2 loop) at the bottom of each panel. We found that 14 of these PH domains have a significantly positively charged region that coincides with the predicted phosphoinositide binding site. Many of these models exhibit the electrostatic sidedness seen in most known PH domain structures (Lemmon and Ferguson, 2000), although some others (such as Osh2p-PH) have an almost completely positive electrostatic profile. Ten of the models with significant positive potential have a highly basic β1/β2 loop (Figure 6A) and four have a basic region that is bracketed by both the β1/β2 and β5/β6 loops (Figure 6B), as observed in the structure of the β spectrin PH domain (Macias et al., 1994). The remaining five models display very weak or no positive potential (Figure 6C). Information on all of these models may be accessed at http://maat.med.cornell.edu/YEAST/Yeast_PH_domains.html.

Nine of the fourteen PH domains predicted to have positively charged ligand binding sites were targeted to the PM or Golgi when analyzed as EGFP or Ras fusion proteins (the Boi2p, Cla4p, Num1p, Opy1p C, Osh1p, Osh2p, Skm1p, Yil105cp, and Ynl047cp PH domains). Seven of the fourteen positive PH domains displayed significant phosphoinositide binding by SPR (including Boi1p-PH, which was not membrane targeted). Only four of the PH domains with strong positive potential (from Bem3p, Osh3p, Ugt51p, and Yhr155wp) failed to show either membrane localization or strong phosphoinositide binding (although Osh3p-PH was nuclear). By contrast, of the five models that do not exhibit significant positive potential, all were for PH domains that were not membrane targeted and did not bind phosphoinositides in SPR studies.

A good correlation therefore emerges between the electrostatic properties of yeast PH domain models and their membrane targeting and/or phosphoinositide binding characteristics. Based solely on their electrostatic properties, our models predicted that 14 yeast PH domains would bind membranes (and/or phosphoinositides). Of these 14, experimental studies independently demonstrated that 10 behave as predicted. Similarly, no membrane binding or targeting was detected for the five PH domains with weak or absent positive potential. As recently described for phospholipase C PH domains

(Singh and Murray, 2003), this analysis therefore appears to have significant predictive value. Equally important, the fact that electrostatic considerations provide such good predictive power argues that delocalized electrostatic attraction plays a critical role in PH domain-mediated membrane targeting events.

Conclusions

Our genome-wide analysis argues that high-affinity and specific phosphoinositide binding is not a common property of *S. cerevisiae* PH domains. In fact, yeast have only one PH domain (from Num1p) with these characteristics. Although we have yet to characterize PtdIns(4,5)P_2 recognition by the Num1p PH domain in structural detail, it appears to resemble PLCδ-PH and may be of value as an additional PtdIns(4,5)P_2 probe. From a functional perspective, the PH domain of full-length Num1p has been shown to be necessary for its localization to cortical patches in yeast cells (Farkasovsky and Kuntzel, 1995). Num1p at the cortex binds tubulin and dynein and is thought to serve as a cortical anchor for dynein as it drives nuclear migration through the bud neck during mitosis (Bloom, 2001).

Most yeast PH domains show no evidence for membrane targeting as isolated domains and bind phosphoinositides too weakly to be measurable using SPR (Table 2). A recent global analysis of yeast protein localization (Huh et al., 2003) suggests that this does not simply reflect our focus on isolated PH domains. Indeed, in that study, only 4 of the 22 intact proteins that contain these 23 PH domains were significantly localized. Seven were unscored or ambiguous in localization; eleven were cytoplasmic; one was at the bud neck; one was mitochondrial; one was at the endoplasmic reticulum; and one was in the cytoplasm, bud, bud neck, and periphery (Table 2). Thus, whether isolated PH domains or intact proteins are considered, bulk localization to cellular membranes does not appear to be an important property of most of these proteins. Elucidating PH domain function in this context will be an interesting challenge.

Two of the most striking conclusions of our study are: (1) that very similar degrees of membrane localization are seen for PH domains with very different phosphoinositide binding affinities (e.g., compare Yil105cp-PH and Num1p-PH) and (2) that quite different localization is seen for PH domains with very similar phosphoinositide binding specificities (e.g., compare Boi2p-PH, Osh1p-PH, and Skm1p-PH).

These conclusions suggest that, while phosphoinositide binding certainly contributes to membrane targeting of most of these PH domains, it does not specify their location. Levine and Munro showed that specific Golgi targeting of the OSBP PH domain (closely related to Osh1p) requires its simultaneous binding to both PtdIns(4)P and another factor (possibly Arf1p) at the Golgi (Levine and Munro, 2002). Our studies in *sac1*Δ yeast support this further. Although they have very similar phosphoinositide binding specificities and affinities, Osh1p-PH recognizes PtdIns(4)P only at the Golgi, whereas Osh2p-PH is recruited to PtdIns(4)P at the Golgi or the PM. In other words, the two PH domains appear to recognize the same lipid but in different contexts.

Among proteins with the PH domain "superfold" (Blomberg et al., 1999), the best-known PH domains (e.g., PLCδ-PH and Grp1-PH) may represent one extreme where the primary (or only) ligand is phosphoinositide. Num1p-PH is the only example of this sort among *S. cerevisiae* PH domains. At another extreme are the PTB, EVH1, and other domains with specific protein ligands that bind in distinct ways. Opy1p C-PH may resemble one of these examples and may have a protein rather than phosphoinositide target. Other yeast PH domains appear to lie between these extremes, perhaps being able to interact with both phosphoinositide *and* protein (or other target). Indeed, simultaneous binding of protein and phosphoinositide ligands to distinct sites has been reported for several domains with the PH domain fold. One example is the PH domain from β-adrenergic receptor kinase (βARK), which binds simultaneously to PtdIns(4,5)P_2 and $G_{\beta\gamma}$ subunits (Lodowski et al., 2003; Pitcher et al., 1995). Another example was provided by recent structural studies of the PTB domains from disabled-1 and disabled-2. The PH domain-like phosphoinositide binding site and PTB domain-like peptide binding site are *both* occupied simultaneously in crystal structures of these domains (Stolt et al., 2003; Yun et al., 2003).

The next phase in analyzing PH domain function in yeast (and humans) is to identify the proposed additional binding partners that define the specificity of membrane localization. In the meantime, the analysis of PH domain function presented here will help direct studies of both phosphoinositide signaling and PH domain-containing proteins in yeast, while also providing a framework for what to expect from the 250 or so examples in the human proteome.

Experimental Procedures

Production and Purification of GST/PH Fusion and Monomeric PH Proteins

To generate GST/PH fusions in *E. coli*, PH domain-encoding DNA fragments were PCR amplified from yeast genomic DNA and subcloned into pGEX-2TK (Amersham-Pharmacia) or pGSTag (Ron and Dressler, 1992). The PH domain boundaries were Ask10p(465-725), Bem2p(1787-1957), Bem3p(632-752), Boi1p(756-906), Boi2p(748-898), Bud4p(1158-1296), Caf120p(61-215), Cdc24p(465-678), Cla4p (58-193), Num1p(2563-2692), Opy1p-N(1-155), Opy1p-C(209-324), Osh1p(267-388), Osh2p(277-398), Osh3p(212-321), Sip3p(308-430), Skm1p(1-132), Spo14p(487-668), Spo71p-N(738-973), Spo71p-C(1022-1241), Syt1p(836-1074), Tus1p(703-883), Ugt51p(234-349), Ybl060wp(384-558), Yhr131cp(155-302), Yhr155wp(305-427), Yil105cp (452-588), Ylr187wp-N(76-232), Ylr187wp-C(243-447), Ynl047cp(429-562), Ynl144cp(170-312), Ypr091cp(109-273), and Ypr115wp(478-731). Site-directed mutagenesis used the QuikChange kit (Stratagene). GST/PH domain fusions were produced and purified as described (Klein et al., 1998).

For generation of untagged or hexahistidine-tagged PH domains in *E. coli*, the PH domains of Boi2p (amino acids 755-891), Cla4p (59-193), Osh1p (279-383), Osh2p (282-389), and Skm1p (1-132) were subcloned into pET11a (Cla4p), pET21a (Boi2p), or pET15b (Osh1p, Osh2p, and Skm1p). Cla4p-PH and PLCδ-PH were purified by cation exchange and gel filtration chromatography (Ferguson et al., 1995). Others were purified by Ni-NTA chromatography (Qiagen) followed by gel filtration.

Dot Blot Overlay Assay

Lipid overlay assays using ^{32}P-labeled GST/PH fusions were performed exactly as described (Kavran et al., 1998).

Surface Plasmon Resonance Analysis of Phosphoinositide Binding

Dioleoylphosphatidylcholine (DOPC) vesicles with or without 3% (mole/mole) added phosphoinositide were prepared and immobilized on L1 sensor chips, and binding experiments were performed as described (Erb et al., 2000; Yu and Lemmon, 2001). Binding to a reference DOPC-only surface was measured simultaneously and subtracted as background. For purified GST/PH fusions, protein concentration was determined by SDS-PAGE analysis and comparison with BSA standards. For quantitative analysis of binding by purified monomeric PH domains (Figure 2B), protein concentration was determined by absorbance at 280 nm using calculated extinction coefficients.

Microscopy

For analysis of PH domain localization in yeast, DNA fragments encoding each PH domain (with the same boundaries used for GST/PH fusions) were subcloned into pGO-GFP (Cowles et al., 1997). For yeast images in Figure 3, these plasmids were transformed into BY4742 using standard methods, cells were prepared as previously described (Audhya and Emr, 2002), and images were collected using a Leica-DMIRBE microscope at 100× magnification and processed using OpenLab deconvolution software (Improvision). For images in Figure 5, the relevant GFP fusion plasmids were transformed into the following strains: *mss4^{ts}*-AAY202 (Stefan et al., 2002), *sjl1*Δ-YCS62 (Stefan et al., 2002), *sac1*Δ-MFY62 (Foti et al., 2001), *stt4^{ts}*-AAY102 (Audhya et al., 2000), *pik1^{ts}*-AAY104 (Audhya et al., 2000), and *stt4^{ts}/pik1^{ts}* (Audhya et al., 2000). Cells were prepared at the permissive and restrictive temperatures as previously described (Audhya and Emr, 2002). Images were collected using a Zeiss Axiovert S1002TV microscope and processed with the Delta Vision deconvolution system.

For expression of EGFP/PH fusions in mammalian cells, PH domain-containing DNA fragments were subcloned into pEGFP-C1 or pEGFP-C3 (Clontech). HeLa cells grown on 35 mm glass bottom dishes (MatTek) were transfected with 5–10 μg of plasmid DNA by calcium phosphate precipitation and imaged 24 hr later using a Leica-DMIRBE microscope at 40× magnification and processed with OpenLab deconvolution software.

Ras Rescue Assay

To facilitate C-terminal fusion of PH domains to RasQ61L(Δf), lacking the farnesylation site, a linker with an internal XhoI site, HA tag, and in-frame stop codon was inserted between the BamHI and NotI sites of p3S0B-SRS (Isakoff et al., 1998) to generate p3S0BL2. PCR-amplified DNA for each PH domain (using the boundaries employed for the most soluble GST fusion) was then subcloned between the BamHI and XhoI sites of p3S0BL2. The resulting plasmids were transformed into *cdc25^{ts}* yeast and transformants grown to mid-log phase in SD (leu dropout) minimal media for several days at room temperature. Cultures were serially diluted (to OD_{600} = 1, 10^{-1}, 10^{-2}, 10^{-3}), and 3 μl of each dilution was spotted in duplicate onto SD (leu dropout) plates. Plates were incubated at the permissive (25°C) or restrictive (37°C) temperature and photographed after 7 to 8 days. Anti-HA immunoblotting confirmed expression of each RasQ61L(Δf)/PH fusion.

Modeling Analysis

Structural templates were chosen from the Protein Data Bank for each PH domain using protein fold recognition programs as described (Singh and Murray, 2003). The sequence to be modeled (the target sequence) and the sequence of its structural template were then aligned based on a combination of (1) the results of local and global (pairwise and multiple) alignment algorithms, (2) alignment of predicted secondary structure elements of the target with known secondary structure of the structural template, and (3) threading analysis.

Implementing these as detailed elsewhere (Singh and Murray, 2003), homology models were constructed by overlaying the target sequence on the template structure according to the optimized sequence alignment. Alignments were further manually edited to produce models that maximized the fitness scores obtained in structure evaluation programs. The illustrations in Figure 6 were generated using GRASP (Nicholls et al., 1991).

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