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Substrate Analysis of the *Pneumocystis carinii* Protein Kinases PcCbk1 and PcSte20 Using Yeast Proteome Microarrays Provides a Novel Method for *Pneumocystis* Signaling Biology.

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Abstract

Pneumocystis carinii (*Pc*) undergoes morphological transitions between cysts and trophic forms. We have previously described two *Pc* serine/threonine kinases, termed PcCbk1 and PcSte20, with PcSte20 belonging to a family of kinases involved in yeast mating, while PcCbk1 being a member of a group of protein kinases involved in regulation of cell cycle, shape, and proliferation. As *Pc* remains genetically intractable, knowledge on specific substrates phosphorylated by these kinases remains limited. Utilizing the phylogenetic relatedness of *Pc* to *Saccharomyces cerevisiae*, we interrogated a yeast proteome microarray containing >4,000 purified protein based peptides leading to the identification of 18 potential PcCbk1 and 15 PcSte20 substrates (Z-score >3.0). A number of these potential protein substrates are involved in bud-site selection, polarized growth, and response to mating α factor and pseudohyphal and invasive growth. Full-length open reading frames suggested by the PcCbk1 and PcSte20 protoarrays were amplified and expressed. These five proteins were used as substrates for PcCbk1 or PcSte20, with each being highly phosphorylated by the respective kinase. Finally, to demonstrate the utility of this method to identify novel PcCbk1 and PcSte20 substrates, we analyzed DNA sequence data from the partially complete *Pc* genome database and detected partial sequence information of potential PcCbk1 kinase substrates PcPx11 and PcInt1. We additionally identified the potential PcSte20 kinase substrate PcBdf2. Full-length *Pc* substrates were cloned and expressed in yeast, and shown to be phosphorylated by the respective *Pc* kinases. In conclusion, the yeast protein microarray represents a novel cross over technique for identifying unique potential *Pc* kinase substrates.

INTRODUCTION

Pneumocystis is a genetically intractable fungal genus causing infection in immunocompromised hosts, with *Pneumocystis jirovecii* representing the species infecting humans and *Pneumocystis carinii* (*Pc*) representing the well-characterized species infecting rodents (Krajicek et al., 2009; Thomas and Limper, 2004; Thomas and Limper, 2007). *P. jirovecii* pneumonia in humans continues to inflict mortality rates ranging between 15-40% (Thomas and Limper, 2004). Most of what is known about host defense and responses to infection with these organisms and all known effective drug regimens have been developed through studies of *Pc*. In particular, immune and cytokine response to *Pc* have been well defined in the host (Carmona et al., 2006; McKinley et al., 2006; Murray and Mills, 1990; Ruan et al., 2008). However, little is known about the actual organism responses to host cell contact or to the lung microenvironment.

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Accumulating evidence indicates that *Pc* adherence to lung epithelial cells promotes organism proliferation and mating responses (Kottom et al., 2003; Limper et al., 1997). Indeed, upon binding of *Pc* to extracellular matrix or to host epithelial cells, an increase in *PcSte20* and *PcCbk1* transcription occurs (Kottom and Limper, 2004; Kottom et al., 2003). The Cbk1 and Ste20 protein kinases have been shown to participate in cell wall remodeling and mating in yeast (Herskowitz, 1995). While the orthologs of these kinases have roles in mating in *S. cerevisiae*, there is not yet direct evidence that they perform a similar function in *Pc*. Verification of a “mating response” in *Pc* will await an in vitro culture system to be tested in a functionally meaningful way. While these initial experiments have given new insight into potential signaling mechanisms of *Pc*, further studies determining the downstream substrates of these two kinases have been severely hampered by the inability to directly manipulate *Pc* genetically. Yet, understanding the signal transduction initiated by the organisms binding to lung epithelium promises to yield key insight into *Pc* biology and the life cycle of this medically important fungal organism.

To circumvent these limitations, our group and others has demonstrated the utility of using heterologous expression of *Pc* genes in phylogenetically related and genetically tractable ascomycetous fungi such as *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (Hauser et al., 2006; Lo Presti et al., 2007; Thomas et al., 1998). Such studies have demonstrated that *Pc* proteins share similar amino acid homology to yeast proteins and can effectively complement and restore a number of defects in respective knockout strains. Thus, yeast represents an important tool to dissect downstream signaling pathways in the non-culturable fungus *Pc*, such as the signaling pathways induced by *Pc* adherence to lung epithelial cells.

During the past decade, DNA microarray technology has provided considerable insight into host-pathogenic interactions. The main application of microarrays in this field has been the study of host responses to various eukaryotic, prokaryotic, and viral pathogens (Eskra et al., 2003; Sandovsky-Losica et al., 2006; Wang et al., 2004). Such studies have led to important advances in understanding of the complex interplay between pathogens and host cells, proving useful in enhancing our understanding at the transcriptional level. However, additional approaches are required to understand regulatory function at the level of protein based cell signaling. In the past, such studies have been an extremely time consuming process as individual candidate proteins are expressed, purifying and characterized in a meticulous stepwise fashion.

In this light, we recently implemented a yeast protein microarray strategy that contained > 4,000 purified protein based peptides from *S. cerevisiae* to determine potentially important substrates for the PcCbk1 and PcSte20, kinases whose expression is induced by binding of the organism to lung epithelium. These studies demonstrate the validity of using this cross species yeast proteome microarray strategy as a novel approach to identify specific *Pc* signal transduction substrates by cloning, expressing, and purifying actual *Pc* orthologues of two identified yeast substrates. In this manner, we were able to determine substrates downstream of two key regulatory kinases present in *Pc*. This strategy provides an alternative approach to study kinases and their related substrates in this genetically intractable fungus.

MATERIALS AND METHODS

Generation of PcCbk1 and PcSte20 protein kinases for probing the yeast proteome microarray

Unless other wise specified, all *P. carinii* organisms and the related *P. carinii* protein kinases were from immune suppressed rat, as we previously described (Kottom and Limper, 2000). To express and purify the PcCbk1 and PcSte20 kinases, the open cDNA reading

frames of the respective genes were subcloned into the yeast expression plasmid pYES2.1 TOPO® (Invitrogen) under the control of the *GALI* promoter (Kottom and Limper, 2004; Kottom et al., 2003). For all reading frames, the stop codon was removed to allow for in-frame translation of an N-terminal V5/6X-His tag present in the plasmid. After overnight induction with 2% galactose, the yeast cells were recovered by centrifugation and re-suspended in Y-PER® reagent (Pierce) in the presence of protease inhibitors (Complete tablets, EDTA-free, Roche Applied Science). Next, the cell suspension was passed through a French Press 2X to rupture the yeast and then clarified by centrifugation at $2500 \times g$ for 10 minutes to remove cell debris. The remaining supernatant was removed and purified using a Y-PER® 6X-His Fusion Protein Purification System (Pierce Chemical Co., Rockford, IL) employing nickel affinity chromatography for protein purification. To verify the presence of the protein after column purification, the purified protein (2.0 µg) was separated on a PAGE gel, transferred to nitrocellulose and probed with an Anti-V5 HRP-conjugated antibody (Invitrogen) at a dilution factor of 1:5000.

Kinase reaction on the microarrays

To screen for potential substrates that are phosphorylated by either PcCbk1 or PcSte20, Protoarray™ Yeast Proteome Microarrays mg v1.0 (Invitrogen, Carlsbad, CA) were probed with the respective kinases according to the manufacturer's protocol. Briefly, 50 nM of either the PcCbk1 or PcSte20 kinase was resuspended in 120 µl of kinase buffer (100 mM MOPS, pH 7.2, 1% Nonidet P40 (NP40), 100 mM NaCl, 10 mg/ml BSA, 5mM MgCl₂, 5mM MnCl₂, 1mM DTT). Next, 1 µl [γ -³²P]-ATP (3000 Ci /mmol, 10 µCi/µl) was added to obtain a final ATP concentration of 33 nM for each array. The final kinase mixture was applied to the array and a coverslip was added on top. Each array was then gently slid into a 50 ml conical tube and capped. As per the manufacturers instructions, an ATP only negative control without kinase was performed in parallel. This ATP only negative control is necessary for each kinase to allow for background subtraction of each spotted array (Kung et al., 2009) The tube was then placed on a flat surface in an incubator set to 30°C for 1 hr without shaking. After 1 hr, the microarray was washed and dried according to protocol, applied to X-ray film, and developed 24 hr later. Both *Pc* kinases were reacted with identical set of arrays in parallel.

Proteome array signal quantification and analysis

Analysis of microarray data was conducted with the help of Invitrogen's data analysis division. GenePix 6.0 software (Molecular Devices) was used to overlay the map of proteins in the array file list to each array image with a fixed feature size of 200 µm (diameter). After aligning each of the 48 subarrays using spots from the autophosphorylating control kinases incorporated into each subarray, pixel intensities for each spot on the array were determined from the software and saved to a text file formatted for use in GenePix.

The GenePix files were next processed using Invitrogen's ProtoArray® Prospector 4.0 analysis software (Invitrogen, Inc.) to determine which proteins were phosphorylated by the respective kinase. The software incorporates nearest neighbor analysis, background correction, Z-score transformation and replica coefficient of variance (CV) filtering as described below. Due to scatter of radioactive detection influencing the signal intensity of nearby spots, a signal scatter correction was performed on raw median spot signals (V) to account for the intensity of neighboring signals as provided by the formula:

$$V_{new} = V_{old} - 0.078 * \sum_i \frac{V_i - B}{R_i^{4+0.2}}$$

where “i” represented all neighbors inside a 5×5 spot square surrounding the spot under investigation, B represented the background calculated as the 10th percentile value of all features of the subarray to which the spot being corrected belongs, and R_i was the distance between features in μm . Background correction was performed for each of the 48 subarrays in each total array. The median signal of negative control features was determined for each subarray. This value was subtracted from the median intensity of a given spot for all yeast protein spots within the subarray.

For each array, a Z-score transformation was applied such that the signal distribution was centered with a mean value of 0 and a standard deviation of 1. For a negative control, significant protein phosphorylations were recorded with “Hit” or “1” where the Z-score was greater than 3, or outside of three standard deviations of the mean value, and the CV between duplicate spots was less than 50%. For assays in which kinase has been exogenously added to arrays, significant phosphorylations recorded by “1” must have had a Z-score greater than 3, a “Signal Used” score greater than 1.5-times the corresponding “Signal Used” of the negative control assay and a replicate coefficient of variance (CV) of less than 50%. Potential substrate proteins had a Z-Factor >0.5 , a confidence interval (CI) P-Value >0.05 , and were confirmed by visual inspection of the array.

PcCbk1 and PcSte20 kinase activity on proteome microarray identified candidate substrates

To verify that the proteins identified by the yeast proteome microarray results could indeed serve as phosphorylation substrates for PcCbk1 or PcSte20, the proteins Clc1 and Dma1 identified from the PcCbk1 screen, and the proteins Lrs4 and YPL257W-A detected in the PcSte20 experiment were amplified from cognate sequences found in the *Saccharomyces* Genome Database <http://yeastgenome.org>, subcloned into pYES2.1 TOPO[®], expressed and purified as described above. These potential substrates were then submitted to phosphorylation assays with the respective *Pc* kinase proteins. To accomplish this, the respective purified yeast substrates (2.0 μg each) were added to 3.0 μg purified PcCbk1 or PcSte20p kinase in kinase buffer (100 mM MOPS, pH 7.2, 1% NP40, 100 mM NaCl, 10 mg/ml BSA, 5mM MgCl_2 5mM MnCl_2) containing 10 μCi [γ -³²P]-ATP (6000 Ci/mmol). Kinase reactions were allowed to run at 30°C for 1 hr. The resulting phosphorylated products were separated by SDS-PAGE and visualized with x-ray film.

Molecular cloning and characterization of the putative PcCbk1 and PcSte20 substrates

After having identified candidate substrates of PcCbk1 and PcSte20 using the yeast proteome array strategy, we next sought to identify these protein orthologues in *Pc*. To accomplish this, analysis of expressed sequence tags from the partially complete *P. carinii* Genome Project (<http://pgp.cchmc.org/>) was undertaken. A keyword search revealed two partial ESTs with significant homology to kinase substrates identified by yeast protoarray. As we searched the databases, we utilized a cutoff of $P < 4\text{e-}16$ on BLASTX analysis comparing the *P. carinii* with the *S. cerevisiae* sequences. For the PcCbk1 and PcSte20 kinases these putative substrates were PcPx11 and PcBdf2, respectively. Using this sequence data, the full-length cDNA's were obtained by 5' and 3' “gene-racing” (GeneRacer[™] Kit, Invitrogen) with either known sense or antisense primers combined with 5' or 3' anchored PCR primers as described previously (Burgess et al., 2008). The derived *P. carinii* sequences were orthologues to the *S. cerevisiae* sequences with the following levels of homology PcPx11 (BLASTX analysis, exact amino acid identities = 34%; positive homologies (exact amino acids + same similar charge amino acids) = 55%; P-value = $4\text{e-}18$) and PcBdf2 (BLASTX, exact amino acid identities = 37%; positive homologies (exact amino acids + same similar charge amino acids) = 52%; P-value= $6\text{e-}23$). These two

sequences were submitted to NCBI and given the final accession numbers EU683040 (PcPx11) and EU683039 (PcBdf2), respectively.

RESULTS

Identification of candidate yeast proteins phosphorylated by the regulatory PcCbk1 or Ste20 kinases

The PcCbk1 and PcSte20p kinases were each expressed in yeast, purified by nickel affinity chromatography and reacted with the yeast proteome peptide microarray in the presence of [γ - 32 P]-ATP. For proper orientation and alignment of the microarray images and subsequent data analysis, the proteome microarray (Invitrogen) contained two fiduciary kinases that had known autophosphorylation activity.

The incubation with the PcCbk1 (Table 1) and the PcSte20 (Table 2) kinases provided distinct and non-overlap phosphorylation patterns on the yeast peptide microarrays, with the exception of the tRNA binding protein ARC1 (Simos et al., 1998). Representative magnified examples of four subarray phosphorylation patterns for each kinase spotted in duplicate are demonstrated in Figure 1. Of note each peptide is spotted in duplicate (boxed), and only positive substrates with reactivity on both duplicate peptide spots were further considered. In addition, the auto-alignment tool using the fiduciary kinases was essential to eliminate occasional background “spots” that did not correspond to any peptide location. Furthermore, an ATP alone treated kinase control microarray was studied in parallel and demonstrated no significant signal at the respective putative kinase substrate spots. After background subtraction, a number of potential kinase substrates were identified for PcCbk1 (Figure 2A) and PcSte20 (Figure 2B) with substantial elevation of phosphorylation signal compared to the negative control array treated with ATP alone, but without kinase.

Candidate yeast substrates identified by the yeast proteome microarray were phosphorylated by the PcCbk1 or PcSte20 protein kinases

We next demonstrated the suitability of our substrate selection strategy by evaluating independent *in vitro* phosphorylation reactions of two putative substrates each for the PcCbk1 (Figures 3) and PcSte20 kinases (Figure 4). For the validation experiments, we selected proteins that were readily expressed and purified. Of the potential PcCbk1 substrates, five of the top seven *S. cerevisiae* substrates were attempted to be purified. Three of them were unable to be tested because little or no expression of the protein occurred in the expression system employed. Two potential PcCbk1 substrates were generated (Clc1 and Dma1) and purified for use in the kinase test reactions. Both of the putative PcCbk1 substrates demonstrated significant phosphorylation by the kinase. For PcSte20, both potential substrates listed (Lrs4 and YPL257W) were successfully purified and phosphorylated by PcSte20. Attempts were made to further purify Glo3, but this yielded unusable quantities for kinase assay. Notably however, in these assays, both kinases effectively phosphorylated the recombinantly expressed purified protein yeast substrates. These reactions provided strong, selective phosphorylation of their microarray-identified protein target, verifying that the initial cross species microarray kinase screening strategy could identify potential target molecules. It remains possible that some potential substrates may not be successfully phosphorylated by the *Pc* kinases due to refolding of the protein during expression compared to the surface bound peptides substrates present on the microarray. Nonetheless, a number of interesting putative substrates were identified using this approach.

The yeast proteome array also identified Bud4 as a potential downstream target of PcCbk1. We have recently independently discovered a Bud4 homolog, which we have termed PcInt1.

PcInt1 is an integrin-like surface molecule that has been implicated in *Pc* interactions with extracellular matrix and organism binding (Kottom et al., 2008). Accordingly, we tested the ability of PcCbk1 to phosphorylate PcInt1 (Figure 3C). The PcInt1 protein was generated and purified in a manner parallel to that described for PcCbk1 and the potential substrate submitted to a kinase reaction, demonstrating that the PcCbk1 could indeed phosphorylate PcInt1.

To further verify the specificity of the PcSte20 and PcCbk1 kinases for these respective substrates, the yeast target proteins identified by the yeast proteome peptide microarrays were subjected to kinase reactions with the opposite kinase in parallel (Figure 5). Cross reactivity between these substrates was not observed. Furthermore, the PcSte20 and PcCbk1 kinases also did not demonstrate any significant auto-phosphorylation. However, we did confirm the functional activity of these kinase proteins, as both the PcSte20 and PcCbk1 enzymes were able to phosphorylate the common kinase substrate PHAS-I (eukaryotic translation initiation factor 4E binding protein 1). We and others have employed this generic substrate to confirm kinase activity for PcCbk1 and PcSte20 (Burgess et al., 2009).

Identification of *Pc* proteins that are phosphorylated by the PcCbk1 and PcSte20 kinases. Finally, to verify that the yeast kinase substrates could be used to identify *Pc* substrates for the respective kinases, a keyword search of the *Pc* genome database was undertaken for all the potential yeast targets. This approach yielded two partial DNA sequences that when translated into protein were found to contain potential kinase substrate sequences. These sequences were identified as PcPx11 for PcCbk1 and PcBdf2 for PcSte20. Using a RACE-based PCR strategy previously described by our group (Burgess et al., 2008) we next proceeded to obtain the full-length cDNAs of both genes (NCBI accession numbers EU683040 for PcPx11 and EU683039 for PcBdf2, respectively.). The proteins encoded by these two sequences were expressed in yeast, purified by nickel affinity chromatography and submitted to phosphorylation reactions using the respective kinases (Figure 6). Significantly, both of the *in vitro* kinase assay with the respective kinases demonstrated substantial specific phosphorylation of the corresponding substrate. To further verify the specificity of each *Pc* kinases (PcCbk1 and PcSte20), each kinase was used in a reaction with the opposite *Pc* substrates previously identified for the other kinase (Figure 6C). Neither PcCbk1 nor PcSte20 demonstrated phosphorylation of the other kinase substrate. Thus, the *Pc* derived kinase substrates appear specific for the respective kinase for which each was identified. This novel proteomic strategy allowed high throughput identification of potential phosphorylation substrates of *Pc* protein kinases.

DISCUSSION

Herein, we report a new method to perform screening identification of potential phosphorylation substrates for life cycle regulatory kinases expressed by *Pc*. The application of the yeast proteome microarray method led to the selection of several novel candidate substrates out of a total set of > 4,000 purified protein based peptides for two key life cycle regulatory kinases, namely the PcCbk1 and PcSte20 protein kinases. Such an approach represents a novel useful strategy to dissect signal transduction cascades present in this genetically intractable, but medically relevant fungal organism.

Using this approach, we identified 18 candidate substrates for the PcCbk1 kinase (Table 1). Interestingly, a number of these potential substrates such as Bud4, Bud14, Px11, and Clc1 are involved in bud-site selection, polarized growth, and response to mating α factor, all responses intrinsic to the yeast Cbk1 pathway. No previous work has been reported describing potential downstream phosphorylation targets for either the *P. carinii* PcCbk1 or the *S. cerevisiae* Cbk1 protein kinases. One prior study performed screening for

phosphorylation targets of yeast kinases using this global screening approach and identified several potential kinase substrates (Ptacek et al., 2005). Unfortunately, this study did not include yeast Cbk1 kinase. This earlier study did include a related protein, named Dbf2. Dbf2p has approx. 33% homology with Cbk1 and both proteins are members of the Ndr family of serine/threonine kinases (Hergovich et al., 2006). In yeast, Dbf2 is involved in transcription regulation, stress responses, and also functions as part of a network of genes involved in mitotic exit (Stoepel et al., 2005). Out of 18 potential PcCbk1 substrates identified from our screen of >4,000 purified protein based peptides, approx. 22% were identical to those previously discovered for yeast Dbf2p (Ptacek et al., 2005). Identical substrates for PcCbk1 and yeast Dbf2 included Vhs2, Bfr1, Drs1, and Arc1.

A second investigation for yeast Dbf2 further verified a number of the substrates we identified in our study, further strengthening the validity of our findings (Mah et al., 2005). These same substrates included Arc1, Smp2, and Vhs2 (Mah et al., 2005). Taken together, these findings provide validity to our peptide microarray experiments in that two similar kinases can indeed recognize specific serine and/or threonine motif(s) present in a given potential substrate. Unfortunately, we were unable to directly compare the *S. cerevisiae* Cbk1 kinase profile to our PcCbk1, as this kinase was not included in the previously published global array of protein phosphorylation in yeast (Ptacek et al., 2005).

Using this proteome array strategy, we also identified 15 additional candidate substrates for the PcSte20 kinase (Table 2). Using the PcSte20 kinase, the substrates Bdf2, Bud27, Glo3, and Vhs2 were identified, which had been shown previously to be involved in the pheromone response, bud site selection and pseudohyphal/invasive growth pathways of yeast Ste20p. Of note, we were able to compare the PcSte20 profile that we obtained to the previous investigations of Zhu *et. al.* as Ste20 kinase was one of the kinases they had previously tested (Zhu et al., 2001). Interestingly, only two of the substrates, Glo3, and Bdf2 were identified substrates for both yeast and PcSte20 kinases. Thus, this approach may be used to detect similarities and divergences in signal transduction pathways across fungal species.

The putative *Pc* kinase substrates identified through this approach provide additional support to the evolving concept that PcCbk1 and PcSte20 are important regulatory kinases acting in signaling cascades controlling key life cycle events. For instance, these substrates include yeast Px11 and Bud4 which are phosphorylated by PcCbk1 kinase. In yeast, PXL1 (paxillin-like) protein participates in maintenance and polarized growth during mating (Gao et al., 2004). Furthermore, yeast PXL1 shows genetic interaction with FKS1, the major beta 1,3-D-glucan synthesis of the yeast cell wall (Mackin et al., 2004). Remodeling of the beta-glucan cell wall is an essential step in morphological alterations and cell cycle traverse of fungi. Furthermore, both *Pc* kinases phosphorylated the yeast substrate Arc1 suggesting a conserved serine/threonine phosphorylation site(s) that is recognized by both kinases. Determination of this motif in *Pc*, may prove a useful strategy to locate other potential substrates of these regulatory kinases within *Pc*.

Due to the difficulties in directly genetically manipulating *Pc*, the use of these proteome microarrays represents a novel strategy for identifying potential kinase substrates in this challenging species. This represents the first such attempt to identify downstream signaling partners in *Pc* using two well-characterized kinases. We believe that the PcSte20 and PcCbk1 kinases are useful enzymes to test this approach, since the catalytic regions of these kinases are well conserved compared to *S. cerevisiae*. For instance, the amino acid sequence of the catalytic domain of PcCbk1 is 75% identical, and the catalytic region of yeast Ste20 is 61% identical to the respective regions in yeast. While it remain possible, and likely, that the substrate specificity of *Pc* will in some aspects differ from that present in *S. cerevisiae*, we

believe that some identified substrates will have sufficient similarity to permit identification through this strategy.

An additional potential downstream substrate in *Pc* identified via the yeast proteome array strategy is Bud4. Bud4 is a potential substrate for the cell cycle kinase Cdc28 in yeast and participates in bud-site selection (Voth et al., 2005). In *Candida albicans*, the Bud4 homolog Int1 has been shown to have homology to human α_M integrin and important in organism binding to the host cell and infection (Gale et al., 1998). Of note, our lab has discovered a Bud4/Int1 homolog, which we have termed PcInt1 with implications in *Pc* binding (Kottom et al., 2008). Specifically, we have demonstrated that the PcInt1 protein is expressed greatest in the *Pc* trophic forms and mediates increased binding to the host extracellular matrix protein fibronectin. Our studies further demonstrate that that purified PcCbk1 can phosphorylate PcInt1 protein. Thus, our observations raises the possibility that PcCbk1 phosphorylation of the PcInt1 may further feedback to regulate the attachment and proliferation of *Pc*, an interesting hypothesis that merits further investigation. Such observations support our contention that the use of this proteome array provides a useful high throughput screen to begin to dissect signaling cascade participating in the life cycle of *Pc*.

We do not know whether all of the *S. cerevisiae* substrates identified by the proteome microarray strategy, will have *Pc* homologs, in part, because *P. carinii* genome is only partially completed. Of the 18 yeast substrates identified using the PcCbk1 kinase, 12 homologs were identified in the partial *Pc* genome database. For PcSte20, of the 15 yeast substrates identified, 6 partial homologs were identified. In contrast, in the work by Ptacek and colleagues, the number of *S. cerevisiae* Ste20 substrates identified was 70 (Ptacek et al., 2005). However, this approach is certainly not comprehensive even under ideal conditions. For instance, even in this prior proteome array of yeast kinase substrates, the ScSte11 substrate was not identified, even though it is a known substrate of ScSte20 (Ptacek et al., 2005; Wu et al., 1995). Thus, this method is viewed as an initial screening approach toward identifying potential downstream substrates engaged in signaling in fungi.

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Figure 1. Representative examples of duplicate peptide spots exhibiting increased phosphorylation signal on microarray peptide spots reacted in the presence of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and buffer containing either **A PcCbk1 or **B**. PcSte20 compared to the concurrent control array reacted in the presence of ATP alone.**

Figure 2. Representative histograms of phosphorylation activity following microarray scanning and background correction for A

PcCbk1 kinase activity. And B. PcSte20 kinase activity. Shown are the respective kinase activities compared to control microarray treated with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ alone in the absence of any kinase.

Figure 3. Recombinantly expressed target yeast substrates are phosphorylated by PcCbk1 **A** Yeast target proteins identified with the proteome peptide microarrays were expressed, purified by nickel affinity chromatography and submitted to Western blotting with anti-V5 antibody recognizing the epitope tag. * Denotes expression of Clc1 and ** denotes expression of Dma1. **B.** The epitope tagged purified yeast proteins were next submitted to phosphorylation reactions in the presence of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and in the presence and absence of the PcCbk1 kinase, demonstrating phosphorylation of the putative yeast kinase substrates. * Denotes phosphorylation of Clc1 and ** denotes phosphorylation of Dma1. **C.** PcCbk1 also exhibits the ability to phosphorylate PcInt1, a homolog of yeast Bud4. Purified PcInt1/V5-tagged protein was used as a kinase substrate for PcCbk1 as described. Lane 1. PcInt1 protein alone. Lane 2. PcInt1 with heat-inactivated PcCbk1. Lane 3. PcCbk1 alone. Lane 4. Active PcCbk1 plus PcInt1. Lane 5. PcCbk1 kinase buffer alone.

Figure 4. Recombinantly expressed target yeast substrates are phosphorylated by PcSte20 **A** Yeast target proteins identified with the proteome peptide microarrays were expressed, purified by nickel affinity chromatography and submitted to Western blotting with anti-V5 antibody recognizing the epitope tag. * Denotes expression of YPL257W and ** denotes expression of Lrs4. **B.** The epitope tagged purified yeast proteins were next submitted to phosphorylation reactions in the presence of [γ - 32 P]-ATP and in the presence and absence of the PcSte20 kinase, demonstrating phosphorylation of the putative yeast kinase substrates. * Denotes phosphorylation of YPL257W and ** denotes phosphorylation of Lrs4.

Figure 5. Both PcCbk1 and PcSte20 kinase fail to phosphorylate the opposite kinase as a substrate
Yeast target proteins identified in the yeast proteome peptide microarrays were subjected to phosphorylation reactions with the opposite kinase to verify the specificity of each kinase for the respective substrate. To further verify that the respective kinase were active, the enzyme was added to a mixture that contained the common kinase substrate, eukaryotic translation initiation factor 4E binding protein 1 (PHAS-I).

Figure 6. Recombinantly expressed target *Pc* substrates identified through the proteome microarray strategy are phosphorylated by *Pc*Cbk1 and *Pc*Ste20 **A** Putative *Pc* target proteins of the *Pc*Cbk1 (*Pc*Px11) and the *Pc*Ste20 (*Pc*Bdf2) kinases identified using the proteome peptide microarrays were expressed, purified and submitted to Western blotting with anti-V5 antibody recognizing the epitope tag. * Denotes expression of *Pc*Bdf2 and ** denotes expression of *Pc*Px11. **B.** The epitope tagged purified yeast proteins were next submitted to phosphorylation reactions in the presence of [γ - 32 P]-ATP and in the presence and absence of the *Pc*Cbk1 or *Pc*Ste20 protein kinases, demonstrating successful phosphorylation of the putative kinase substrates. * Denotes phosphorylation of *Pc*Bdf2 and ** denotes phosphorylation of *Pc*Px11. **C.** To further verify the specificity of both *Pc* kinases, each kinase was used in a reaction with the opposite *Pc* substrates as shown in B. Neither *Pc*Cbk1 nor *Pc*Ste20 phosphorylated the other kinase substrate. Thus, the *Pc* derived kinase substrates appear specific for the respective kinase for which each was identified.

Table. 1

Candidate substrates identified for PcCbk1 kinase.

ORF	Common name	Description	CV	Z-Score
YGR167W	Clc1	Clathrin light chain, subunit of the major coat protein involved in intracellular protein transport and endocytosis; thought to regulate clathrin function (Nelson and Lemmon, 1993; Silveira et al., 1990)	6%	11.50
YOR239W	Abp140	Nonessential protein that binds actin filaments and localizes to actin patches and cables, has similarity to S-adenosylmethionine (AdoMet)-dependent methyltransferases (Asakura et al., 1998; Katz et al., 2003)	19%	8.16
YLR150W	Stm1	Translation initiation factor eIF-4B, has RNA annealing activity; contains an RNA recognition motif and binds to single-stranded RNA (Altmann et al., 1995; Coppolecchia et al., 1993)	24%	6.19
YKR079C	Trz1	tRNase Z, involved in RNA processing, has two putative nucleotide triphosphate-binding motifs (P-loop) and a conserved histidine motif, homolog of the human candidate prostate cancer susceptibility gene ELAC2 (Chen et al., 2005)	6%	5.23
YGL056C	Sds23	One of two <i>S. cerevisiae</i> homologs (Sds23p and Sds24p) of the <i>Schizosaccharomyces pombe</i> Sds23 protein, which genetic studies have implicated in APC/cyclosome regulation (Jones et al., 2000)	3%	4.96
YGL105W	Arc1	Protein that binds tRNA and methionyl- and glutamyl-tRNA synthetases (Mes1p and Gus1p), delivering tRNA to them, stimulating catalysis, and ensuring their localization to the cytoplasm; also binds quadruplex nucleic acids (Frantz and Gilbert, 1995; Galani et al., 2001)	4%	4.75
YNL116W	Dma1	Protein involved in ubiquitin ligation; plays a role in regulating spindle position and orientation; functionally redundant with Dma2p; orthologous to human RNF8 protein, also has sequence similarity to human Chfr (Fraschini et al., 2004; Loring et al., 2008)	4%	4.54
YAR014C	Bud14	Protein involved in bud-site selection, Bud14p-Glc7p complex functions as a cortical regulator of dynein; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern (Knaus et al., 2005)	4%	4.42
YIL091C		Protein required for cell viability, uncharacterized	5%	4.39
YNL186W	Ubp10	Ubiquitin-specific protease that deubiquitinates ubiquitin-protein moieties; may regulate silencing by acting on Sir4p; involved in posttranscriptionally regulating Gap1p and possibly other transporters; primarily located in the nucleus (Kahana, 2001; Singer et al., 1998)	3%	4.26
YJR092W	Bud4	Protein involved in bud-site selection and required for axial budding pattern; localizes with septins to bud neck in mitosis and may constitute an axial landmark for next round of budding; potential Cdc28p substrate (Chant and Herskowitz, 1991; Sanders and Herskowitz, 1996)	10%	4.11
YIL135C	Vhs2	Cytoplasmic protein of unknown function; identified as a high-copy suppressor of the synthetic lethality of a <i>sis2 sit4</i> double mutant, suggesting a role in G1/S phase progression; similar to Mif3p (Munoz et al., 2003)	5%	3.82
YLR331C	Jip3	Uncharacterized protein	5%	3.65
YOR198C	Bfr1	Component of mRNP complexes associated with polyribosomes; implicated in secretion and nuclear segregation; multicopy suppressor of BFA (Brefeldin A) sensitivity (Jackson and Kepes, 1994)	8%	3.28
YGR021W		Putative protein of unknown function	3%	3.21
YLL008W	Drs1	Nucleolar DEAD-box protein required for ribosome assembly and function, including synthesis of 60S ribosomal subunits (Adams et al., 2002)	34%	3.17
YMR165C	Smp2	Mg ²⁺ -dependent phosphatidate (PA) phosphatase, catalyzes the dephosphorylation of PA to yield diacylglycerol and P _i , responsible for de novo lipid synthesis; homologous to mammalian lipin (Han et al., 2006; Irie et al., 1993)	5%	3.06
YKR090W	Px11	LIM domain-containing protein that localizes to sites of polarized growth, required for selection and/or maintenance of polarized growth sites, may modulate signaling by the GTPases Cdc42p and Rho1p; has similarity to metazoan paxillin (Mackin et al., 2004)	15%	3.04

Table 2

Candidate substrates identified for PcSte20 kinase.

ORF	Common name	Description	CV(%)	Z-Score
YGR126W		Function unknown	3.28	37.1
YER122C	Glo3	ADP-ribosylation factor GTPase activating protein (ARF GAP), involved in ER-Golgi transport; shares functional similarity with Gcs1p (Moss and Vaughan, 1998)	4.23	27.4
YER079W		Hypothetical protein	3.09	17.2
YDL025C		Putative protein kinase, potentially phosphorylated by Cdc28p (Ubersax et al., 2003)	3.28	16.7
YDL070W	Bdf2	Protein involved in transcription initiation at TATA-containing promoters; associates with the basal transcription factor TFIID; contains two bromodomains; corresponds to the C-terminal region of mammalian TAF1; redundant with Bdf1p (Matangkasombut et al., 2000)	3.66	12.4
YGL153W	Pex14	Peroxisomal membrane peroxin that is a central component of the peroxisomal protein import machinery (Heiland and Erdmann, 2005)	35.6	10.02
YPR137C-A		Transposable element	39.1	7.05
YFL023W	Bud27	Protein involved in bud-site selection, nutrient signaling, and gene expression controlled by TOR kinase; diploid mutants show a random budding pattern rather than the wild-type bipolar pattern; plays a role in regulating Ty1 transposition (Ni and Snyder, 2001; Nyswaner et al., 2008)	31.8	4.36
YDR439W	Lrs4	Protein involved in rDNA silencing; positively charged coiled-coil protein with limited similarity to myosin (Smith et al., 1999)	14.7	4.16
YPL-257W-A		Transposable element	15.4	4.09
YDR153C	Ent5	Protein containing an N-terminal epsin-like domain involved in clathrin recruitment and traffic between the Golgi and endosomes; associates with the clathrin adaptor Gga2p, clathrin adaptor complex AP-1, and clathrin (Duncan et al., 2003; Wendland et al., 1999)	14.5	4.01
YDR034C-C		Transposable element	16.2	3.17
YNL054W-A		Transposable element	34.0	3.05
YGL105W	Arc1	Protein that binds tRNA and methionyl- and glutamyl-tRNA synthetases (Mes1p and Gus1p), delivering tRNA to them, stimulating catalysis, and ensuring their localization to the cytoplasm; also binds quadruplex nucleic acids (Frantz and Gilbert, 1995; Galani et al., 2001; Moss and Vaughan, 1998)	4.2	2.56
YIL135C	Vhs2	Cytoplasmic protein of unknown function; identified as a high-copy suppressor of the synthetic lethality of a sis2 sit4 double mutant, suggesting a role in G1/S phase progression; similar to Mif3p (Huh et al., 2003; Munoz et al., 2003)	2.92	1.8