Development and Validation of Kinase Substrate Screening on ProtoArray™ High-Density Protein Microarrays

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Abstract

Identifying biologically relevant substrates for protein kinases is a critical step in understanding the function of these clinically important enzymes. Traditional approaches for kinase substrate identification are expensive, slow, and lack sensitivity. For this reason, many kinase activity assays employ generic substrates or peptides that decrease the reliability of these assays for drug development. We describe here the development and validation of a rapid and sensitive microarray-based kinase substrate identification technology, which enables parallel screening of kinases against thousands of potential native protein substrates. This paper describes the validation of this approach and use of the resulting data for pathway mapping.

Introduction

Protein kinases play a central role in the regulation of multiple cellular processes and in diseases; in fact, 244 kinases have been mapped to disease loci (1). It is not surprising, therefore, that a large number of biotech and pharmaceutical companies are seeking to discover and bring to the clinic compounds that demonstrate specific inhibition of kinases involved in disease. Some examples of kinase inhibitors already in clinic include Gleevec® (Novartis), an Abl and c-Kit kinase inhibitor that has been successful in the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors, and Herceptin® (Genentech), an antibody that targets the HER2/neu (erbB2) protein for treatment of breast cancer. The family of human protein kinases consists of more than 500 members of which only a fraction have been characterized to date. Much is still not known about the biological function of many kinases, the protein substrates that are phosphorylated by these kinases, or the roles of these kinases and substrates in disease.

The importance of protein kinases in virtually all processes regulating cell transduction illustrates the potential for kinases and their cellular substrates as targets for therapeutics. Considerable efforts have been made to elucidate kinase biology by identifying the substrate specificity of kinases and using this information for the prediction of new substrates. Some of the approaches used to date include creation of a database from annotated phosphorylation sites, prediction of substrate sequence patterns from available structures of kinase/peptide substrate complexes, and screening of peptide libraries and peptide arrays (2,3). More recent efforts include attempts to map the phosphoproteome using mass spectroscopy-based techniques. While these studies have provided some information about kinase biology, they have been severely limited by their complexity, expense, lack of sensitivity, the use of non-structured peptides, and by poor representation of potential substrates in the screens.

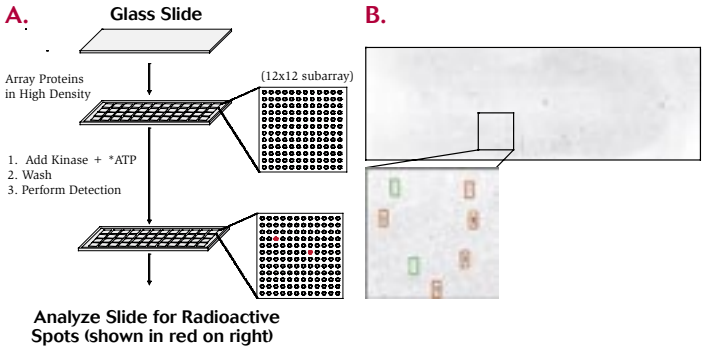
Invitrogen is pioneering the use of arrays of whole or partial proteomes to improve the success rates of drug discovery. This report describes how ProtoArray™ technology rapidly converts gene sequences into arrays of functional proteins that can be used to reveal new disease pathways and define the specificity and selectivity of potential drugs. In addition, this paper discusses how the ProtoArray™ high-density protein microarray technology is an ideal format for identifying biologically relevant substrates for protein kinases in a rapid, cost-effective, and comprehensive fashion.

Validation results

ProtoArray™ technology enables fast, simple, and comprehensive kinase substrate screening.

Each ProtoArray™ microarray contains thousands of *S. cerevisiae* or *H. sapiens* proteins spotted in high density on glass slides. These slides can be probed to identify protein interactions with DNA, proteins, lipids, sugars, small molecules, and enzymes. The first proof-of-principle experiment demonstrating that these arrays can be used to reveal substrates of proteins kinases was carried out on the Yeast ProtoArray™ microarray, which contains over 4000 unique yeast proteins spotted in duplicate. The experimental outline is simple (Figure 1A). A solution comprising a kinase and radioactive ATP was incubated on a Yeast ProtoArray™ microarray, and then the slide was washed and exposed to a phosphoimager (Figure 1B). The experiment identified 41 proteins specifically phosphorylated by the exogenous kinase.

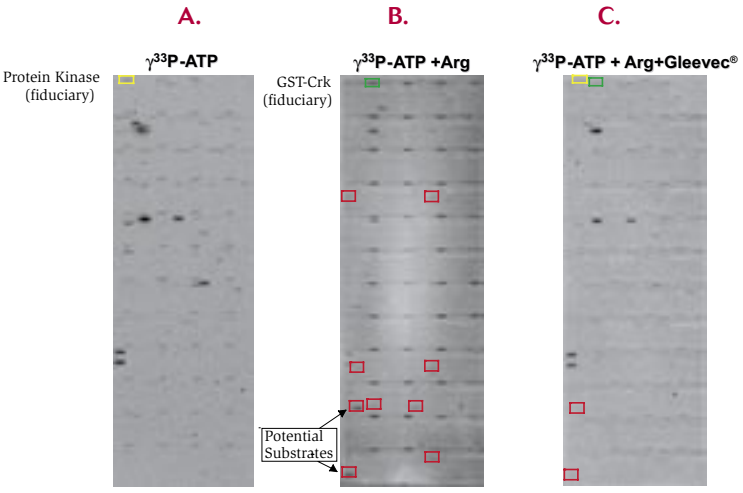
Figure 1. Kinase-substrate assay on the Yeast ProtoArray™ Microarray.



A) Experimental design of substrate screening assay. B) The Yeast ProtoArray™ microarray containing > 4000 different yeast proteins probed with a purified kinase. Inset: positives boxed in green (autophosphorylation) and red (substrates).

Initial work with Human ProtoArray™ microarrays demonstrates kinase substrate discovery value. To test our platform for identification of kinase substrates, we chose the human protein kinase Arg. This kinase, along with its closely related homolog Abl, is known to be involved in the etiology of chronic myeloid leukemia (CML) and is a target for the anti-cancer agent Gleevec®. Human ProtoArray™ microarrays were manufactured with 1500 different quality-controlled recombinant human proteins produced in Invitrogen's proprietary high-throughput insect cell expression and parallel purification systems. A known Abl/Arg substrate, Crk, was printed in regular intervals on the array as a positive control. The Human ProtoArray™ microarray in Figure 2A was incubated with radiolabeled ATP alone; proteins that show a signal on this array are kinases present on the array that autophosphorylate. The array in Figure 2B was incubated with Arg in the presence of radiolabeled ATP. This kinase phosphorylated the control substrate Crk in every subarray; in addition, nine other proteins, that did not give signal with ATP alone, were observed to be phosphorylated in the presence of Arg. We also looked at the effect of adding an Arg/Abl kinase-specific inhibitor and found that the inhibitor specifically decreased phosphorylation of Crk and the nine other microarray identified substrates (Figure 2C), confirming that these proteins were phosphorylated by Arg kinase.

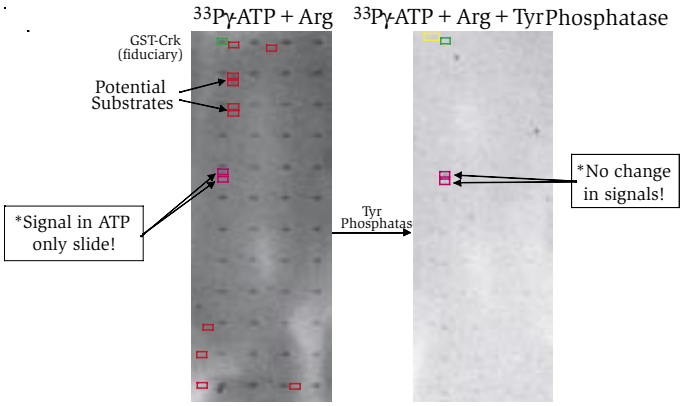
Figure 2. Identification of substrates for Arg kinase.



ProtoArray™ microarrays containing 1500 different human proteins were treated with ATP (A), ATP and Arg (B), or ATP, Arg, and Arg-specific inhibitor (C). Nine substrates were identified for Arg (boxed in red).

Verification of specific phosphorylation by a human kinase. Arg kinase is known to specifically phosphorylate tyrosine residues on certain proteins. To verify that Arg kinase maintains this specificity for tyrosine residues in array-based experiments, Human ProtoArray™ microarrays were treated sequentially with Arg kinase followed by a phosphotyrosine phosphatase. As shown in Figure 3, all proteins phosphorylated by Arg kinase on the array are dephosphorylated by the phosphotyrosine phosphatase, confirming that Arg kinase substrates on the array are appropriately phosphorylated on tyrosine residues. Signals from proteins that autophosphorylate (*i.e.*, that show signal in the absence of exogenous kinase) were not affected by phosphotyrosine phosphatase treatment, indicating that these were kinases that autophosphorylate serine/threonine residues.

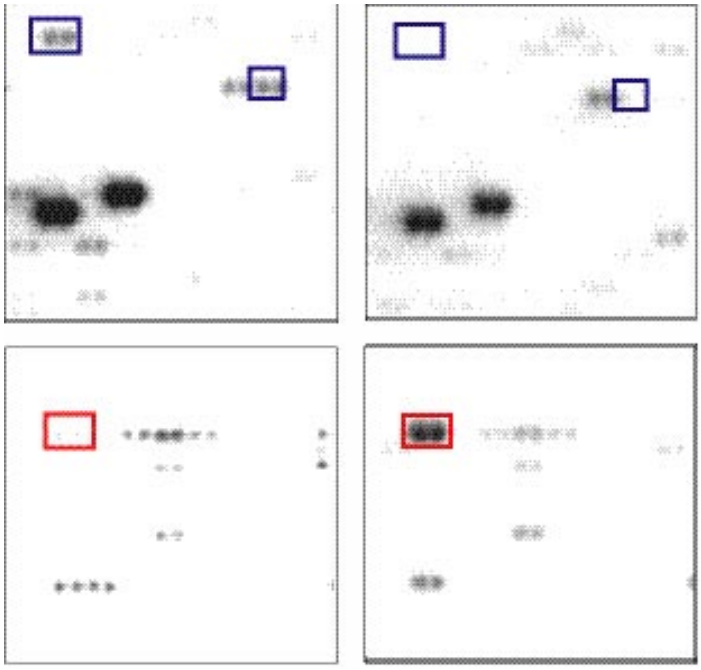
Figure 3. Phosphotyrosine phosphatase reduces Arg substrate phosphorylation.



A Human ProtoArray™ microarray containing the eight identified Arg substrates was probed with Arg kinase and then subsequently with a phosphotyrosine phosphatase.

Substrate phosphorylation is kinase-specific. The results with Arg kinase on Human ProtoArray™ microarrays clearly demonstrated that this kinase is highly selective in the protein substrates that it phosphorylates. In order for this application of the ProtoArray™ technology to be useful to a wide range of kinase biologists, the ability to distinguish phosphorylation patterns of different kinases must be established. Consequently, ProtoArray™ microarrays printed with 2500 different human proteins were incubated with ^{33}P-ATP and either Arg or PKC kinase (Figure 4) or with ^{33}P-ATP alone (not shown). As shown in Figure 4, phosphorylation signals specific to each kinase were clearly observed. The majority of signals present in both experiments were due to autophosphorylation by some of the ~400 kinases printed on the array. Analysis of the whole array revealed dozens of proteins that were specific to one of the kinases. We have now characterized the phosphorylation patterns of over a dozen different human kinases and have identified large numbers of unique substrates for each kinase.

Figure 4. Specificity of kinase phosphorylation on Human ProtoArray™ microarrays.

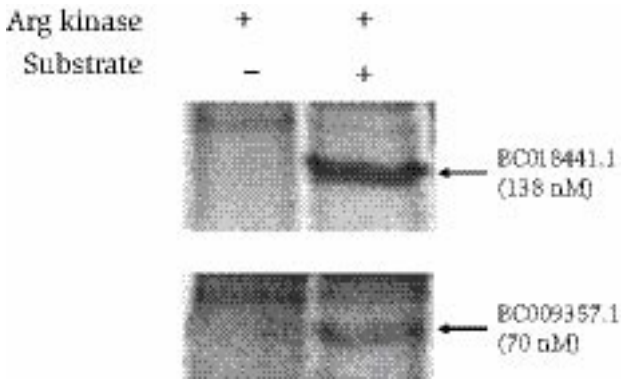


Two Human ProtoArray™ microarrays were incubated with ^{33}P-ATP and either Arg (left column) or PKC kinase (right column). Two representative subarrays are shown: In subarray 1 (top row), two proteins phosphorylated specifically by Arg kinase are boxed in blue; in subarray 2 (bottom row), a protein phosphorylated specifically by PKC is boxed in red.

Validation of substrate identification in an independent assay. Biochemical validation of the array-based substrate screening assay was initially carried out by determining whether proteins phosphorylated by Arg kinase on the array would also be phosphorylated in a different assay format. Figure 5 shows the results of assays in which two of the substrate proteins were incubated in solution with Arg kinase in the presence of radiolabeled ATP. Separation of the reaction mixtures on denaturing gels demonstrated that proteins at the expected molecular weight of the substrate proteins were indeed phosphorylated in solution. These results strongly suggest that these proteins maintain their native conformation on the array, allowing them to be phosphorylated by specific kinases.

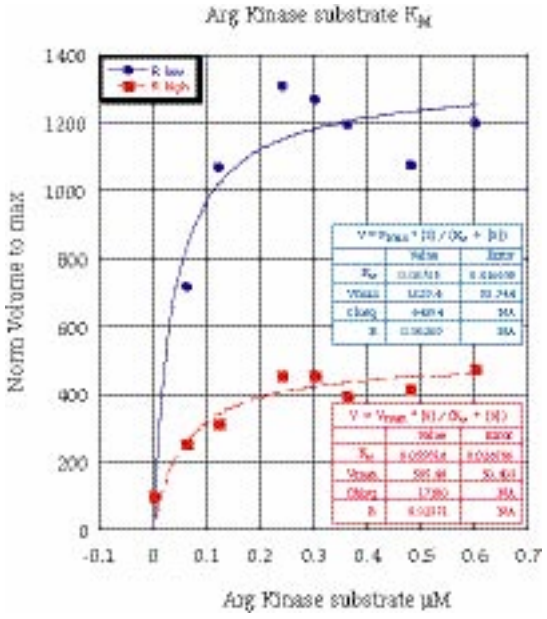
Detailed validation studies reveal the highest affinity substrate for a pharmacologically relevant kinase reported to date. Although phosphorylation of proteins by kinases in experiments, such as the one shown in Figure 5, is a prerequisite for identifying substrates for these enzymes, additional lines of evidence are needed to demonstrate physiological relevance. One such line of evidence is data showing that the substrate is phosphorylated at concentrations likely to occur in a cell. One of the eight proteins identified on the ProtoArray™ microarray as a substrate for Arg kinases was selected for more detailed K_M measurements based on the protein's known role in cell division. Analysis of the data from this experiment yields a K_M for the substrate of approximately 50 nM (Figure 6). Not only is this value well within a potential intracellular concentration for a protein, but it is also lower than any K_M value previously reported for Arg kinase.

Figure 5. Arg kinase phosphorylation of substrate in solution.



Arg kinase alone or mixed with substrate proteins was incubated at 30°C for 30 minutes and then run on an SDS-PAGE gel and phosphoimaged.

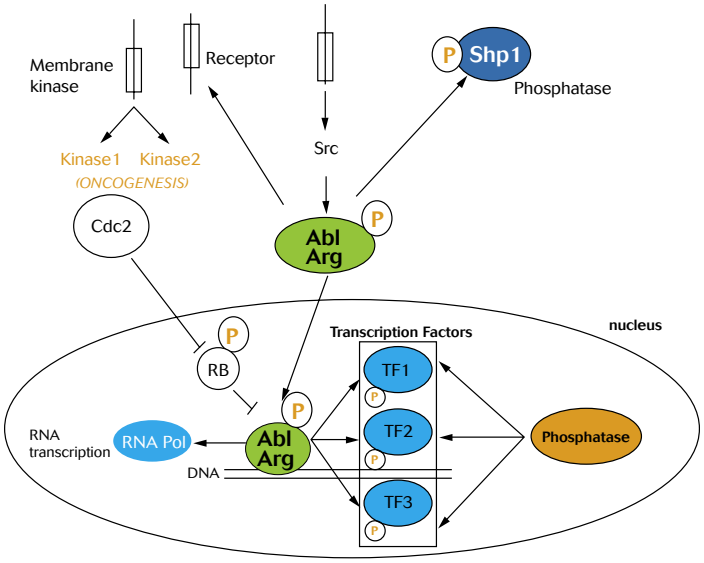
Figure 6. K_M determination for an Arg kinase substrate identified on the ProtoArray™ microarray.



Arg kinase was incubated with different concentrations of the substrate protein and the phosphorylation of the protein was measured in a gel-based assay similar to the one shown in Figure 4.

ProtoArray™ data is used to generate a new kinase pathway. In addition to biochemical validation, it is also desirable to see concordance of ProtoArray™ results with published data. In fact, a search of the literature and publicly available databases revealed that one of the proteins proven to be a substrate for Arg Kinase on a Human ProtoArray™ microarray, Shp1, had indeed been annotated as a substrate for this kinase. Using a protein-protein interaction assay on a Human ProtoArray™ microarray, we also demonstrated for the first time that Arg kinase forms a stable interaction with Shp1 (data not shown). Shp1 is a phosphotyrosine phosphatase localized at the plasma membrane; our data, as well as the published data, are therefore consistent with co-localization and co-regulation of Shp1 phosphatase and Arg kinase (Figure 7). Other published reports indicate that following activation by Src, Arg and Abl kinases translocate into the nucleus, although the functional consequences of this translocation have not been clarified. ProtoArray™ results, however, clearly showed that these kinases phosphorylated several transcription factors that may have roles in cell cycle function. An RNA polymerase was also phosphorylated, providing another line of evidence that these kinases regulate RNA transcription and gene expression. Equally intriguing is the finding that a membrane-associated receptor present on the array was phosphorylated by Arg kinase. Interaction of this receptor with a membrane-associated kinase has been shown by others to result in the activation of two kinases that have been implicated in oncogenesis. This finding represents a new and potentially therapeutically relevant link between the Arg/Abl kinases and cancer.

Figure 7. Pathway mapping with Arg kinase-substrate ProtoArray™ data.



Conclusion

We have combined unprecedented protein content with a simple-to-use microarray assay to generate new knowledge about protein kinases with unequalled efficiency. We have demonstrated specific phosphorylation of both known and novel substrates using Human and Yeast ProtoArray™ high-density protein microarrays and have validated these proteins as substrates using more standard assays. Combining this new type of information with Invitrogen's other capabilities for measuring phosphatase activity, protein-protein interactions, and drug inhibition on microarrays allows scientists to link kinases to intracellular signaling networks and generate new understandings about kinases and their substrates as drug targets with unmatched speed and efficiency.

Implications of ProtoArray™ technology

The discovery of new kinase substrates by Invitrogen and its collaborators using the ProtoArray™ technology platform demonstrates the enormous value of high-content protein arrays. This was clearly illustrated in experiments using Arg kinase: nine substrates were identified using an array printed with 1500 human proteins, but six more were found using a 2500 protein array. Extrapolating to an array containing a representative protein from the approximately 30,000 human genes (the UniProteome) suggests that over 150 substrates would be identified, thereby greatly increasing the informational value of the experiment.

References

1. Manning, G., Whyte, D.B., Martiniez, R., Hunter, T., and Sudarsanam, S *(2002)*. The protein kinase complement of the human genome. *Science 298, 1912-1934.*
2. Kreegipuu, A., Blom, N., Brunak, S. and Jarv, J *(1998)* The statistical analysis of protein kinase specificity determinants. *FEBS Letters 430, 45-50.*
3. Rychlewski, L., Kschischo, M., Dong, L., Schutkowksi, M., and Reimer, U. *(2004)* Target specificity analysis of the Abl kinase using peptide microarray data. *J. Mol. Biol. 336, 307-311.*

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