Substrate Fingerprinting

DOI: 10.1002/anie.200703473

Peptide Microarray for High-Throughput Determination of Phosphatase Specificity and Biology**

*Hongyan Sun, Candy H. S. Lu, Mahesh Uttamchandani, Yun Xia, Yih-Cherng Liou, and Shao Q. Yao**

Reversible phosphorylation of proteins (a critical posttranslational event catalyzed by kinases and phosphatases) occurs in >20% of human proteins.[1] Although kinases have been studied extensively,[2] knowledge about the biological characterization of phosphatases, for example, their cellular partners and substrate specificities, is limited.[3] The reason is, in part, the lack of strict substrate specificity in many phosphatases, as well as a shortage of techniques that enable quick and accurate determination of potential phosphatase substrates. There are two main classes of protein phosphatases, namely tyrosine phosphatases (PTPs) and Ser/Thr phosphatases. Most approaches developed thus far have focused on mapping the substrate specificity of PTPs.[4–6] Early studies involved kinetic assays of individually synthesized phosphopeptides.[4] More recently, combinatorial peptide libraries and SPOT synthesis were introduced.[5] In the latest example, Waldmann and co-workers introduced a peptide-microarray strategy for mapping the substrate specificity of PTPs[6] by immobilizing phosphopeptides on glass slides and detecting their dephosphorylation with a fluorescently labeled anti-*p*Tyr antibody; the strategy is a major improvement over previous membrane-based methods.[7] The main drawback, however, is that it can only study PTPs due to the lack of reliable anti-*p*Ser/Thr antibodies. Herein, we report the first peptide microarray for high-throughput studies of Ser/Thr phosphatases.

A peptide microarray is a miniaturized screening platform that enables thousands of individually addressable peptides to be simultaneously interrogated on a glass slide.[8] Over the last few years, the peptide microarray has become a viable choice for high-throughput determination of enzyme substrate specificities.[9] In our current work, we show that glass slides that have multiple peptide substrates of Ser/Thr phosphatases immobilized on them can be used to simultaneously determine the preference of the enzymes for the different substrates; with this information, new biology may be discovered. In contrast to the approach of Waldmann and co-workers, our method is amenable to both Ser/Thr phosphatases and PTPs. Compared to other approaches, it offers unique advantages in terms of throughput and sensitivity.[10]

We synthesized 87 putative peptide substrates of Ser/Thr phosphatases, each containing 11 amino acid residues with a centrally located *p*(Ser/Thr)–Pro moiety flanked by 5 and 4 amino acids on each side (Figure 1a). Protein phosphorylation/dephosphorylation on (Ser/Thr)–Pro moieties is highly prevalent amongst known kinase/phosphatase substrates. These peptide sequences were extracted from putative protein substrates of the peptidyl prolyl *cis*/*trans* isomerase (PPIase) or Pin1. Pin1, together with its regulatory kinases/phosphatases, is a key cell-regulatory protein.[11] Many physiological substrates of Pin1 are known. Enzymes that control phosphorylation/dephosphorylation of these sub-

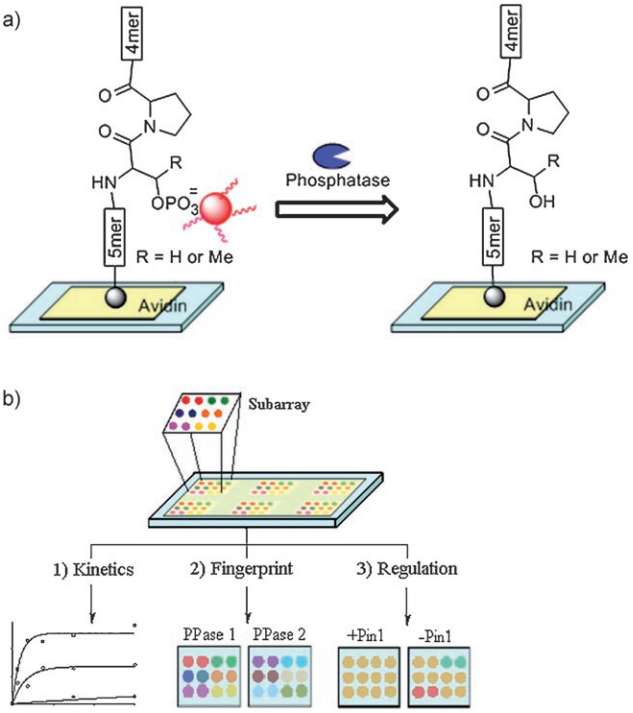

Figure 1. a) The *p*(Ser/Thr)–Pro peptide microarray used to target Ser/Thr phosphatases. b) The subarray format and three types of applications introduced in the current study.

[*] H. Sun, Prof. Dr. S. Q. Yao
Departments of Chemistry
National University of Singapore
3 Science Drive 3, Singapore 117543 (Singapore)
Fax: (+65) 6779-1691
E-mail: chmyaosq@nus.edu.sg
C. H. S. Lu, M. Uttamchandani, Y. Xia, Prof. Dr. Y. Liou, Prof. Dr. S. Q. Yao
Department of Biological Sciences
National University of Singapore
14 Science Drive 4, Singapore 117543 (Singapore)

[**] Funding support was provided by the National University of Singapore (NUS) and the Agency for Science, Technology and Research (A*STAR) of Singapore.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

strates, however, have not been well studied. In fact, PP2A is the only Pin1-regulated phosphatase identified to date.[12] Our aims in this work were therefore to develop a peptide microarray that allows accurate determination of the activities of candidate Ser/Thr phosphatases against putative Pin1 peptide substrates and to identify unknown physiological phosphatase/substrate pairs whose activity is regulated by Pin1 *cis*/*trans* isomerization. The peptides were individually synthesized by standard solid-phase peptide chemistry and characterized by LC-MS. An extra hydrophilic linker and biotin were introduced to the N terminus of each peptide, which was then subsequently immobilized onto an avidin-coated glass slide to generate the corresponding peptide microarray.

To detect peptide dephosphorylation on the glass slide, we used a signal-decrease assay with the commercially available Pro-Q Diamond dye, which is capable of fluorescence-based measurement of *p*Ser/Thr/Tyr residues present in peptides and proteins (Figure 1a).[13] Compared to other microarray-based methods that report phosphorylation/dephosphorylation,[6,9b,c] the Pro-Q method is advantageous in terms of its safety, ease of handling, and sensitivity.[13] On each peptide microarray, 6 identical subarrays were fabricated, with each containing all 87 putative Pin1 peptides individually spotted in duplicate (Figure 1b). Each subarray comprises approximately 200 different spots, thereby allowing up to 1200 different enzymatic assays to be simultaneously performed on the same slide. By closely monitoring the dephosphorylation event of every peptide in the whole collection (Figure 1b), one can 1) carry out kinetic measurements and obtain the relative rates of dephosphorylation (k_{obs}) for many peptides against a phosphatase, 2) generate unique and comparative substrate "fingerprints" of different phosphatases, and 3) discover potential phosphatase/substrate pairs regulated by Pin1. To ensure the accuracy of our results, we determined the variability of spots from different subarrays; a high correlation (Pearson coefficient $r > 0.9$) in fluorescence reading was obtained for the same peptides spotted across different subarrays. We further confirmed that the fluorescence reading was linearly proportional to the amount of phosphopeptide present in each spot. Nonphosphorylated peptides did not give any noticeable fluorescence. We noticed, however, that different peptides spotted on the same (sub)-array, despite a uniform spotting concentration, registered varying degrees of fluorescence reading after Pro-Q treatment (Figure 2a). This could be due to the presence of different hydrophobic environments around the phosphoamino acid in the peptides. The same phenomenon had been reported previously.[13]

We next investigated whether the peptide microarray could be used for simultaneous kinetic studies of a Ser/Thr phosphatase. Lambda phosphatase,[14] an enzyme possessing Ser/Thr phosphatase activity from bacteriophage λ and distantly related to PP2A and PP1 (also a Ser/Thr phosphatase), was used as the model protein. Both concentration- and time-dependent experiments were carried out by applying different amounts of the enzyme (0–8 U; Figure S9 in the Supporting Information) to different subarrays on the glass slide, as well as incubating the subarrays for different periods of time

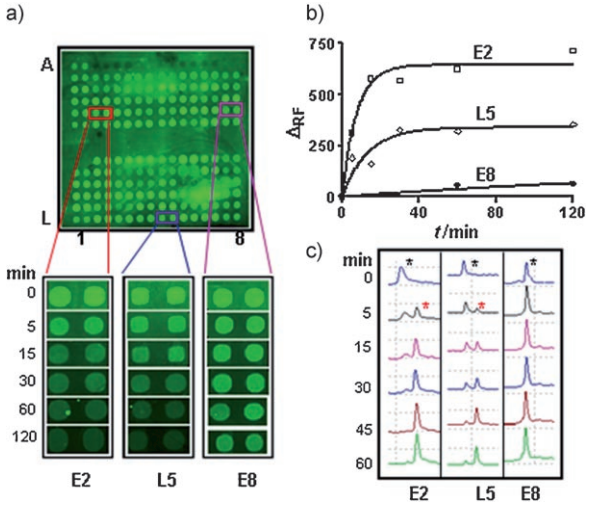

Figure 2. a) Time-dependent experiment with Lambda phosphatase. The six subgrids were incubated with Lambda phosphatase at different time intervals. Only one subarray is shown (0 min). The "expanded" images represent time-dependent results of three peptides (**E2**, **L5**, & **E8**). Samples were spotted in duplicate. b) The kinetic profiles and fitted curves of three representative peptides, **E2** (□), **L5** (◇), and **E8** (●). c) The time-dependent HPLC assay validation experiment of the three peptides. No noticeable dephosphorylation of **E8** was detected. ⋆: starting peptides; ⋆: dephosphorylated peptides.

(Figure 2a). All experiments were performed in triplicate. Concomitant fluorescence decreases were observed with increasing enzyme concentrations and incubation times ("expanded" images in Figure 2a) for all active peptide spots (that is, spots that show a decrease in fluorescence reading upon treatment with Lambda phosphatase). An optimized enzyme concentration was thus obtained and used for all subsequent experiments. Inhibition experiments were carried out by preincubating the Lambda phosphatase with increasing amounts of Na_3VO_4 (a general phosphatase inhibitor) before it was applied to the peptide microarray; a complete abolishment of phosphatase activity was observed with 1.4 mM Na_3VO_4, a result indicating the specific detection of phosphatase activity on the microarray. To obtain kinetic data, the fluorescence data from the time-dependent experiments, representing a total of 1044 individual data points (87 peptides × 6 time intervals, in duplicate) were quantified, filtered, and fitted to obtain the relative rates of dephosphorylation, k_{obs}, where $k_{obs} = (k_{cat}/K_M) \times E$ and represents the observed rate of catalysis/Michaelis constant (k_{cat}/K_M) ratio under a given enzyme concentration (E). Representative fitted curves are shown in Figure 2b. Since our assay was run under conditions in which the enzyme and peptide substrate concentrations were well below the K_M value, the k_{obs} values thus truly reflect the k_{cat}/K_M ratios[9d,15] and can be used to compare the kinetic profiles of different substrates for the same enzyme. To ensure the quality of the k_{obs} values obtained, we subjected the data to stringent filtering criteria ($r > 0.85$ in fitted curves) and significant dephosphorylation signals (> 40% signal decrease from the original Pro-Q readings), and we were able to obtain reasonable k_{obs} values for 53 of the 87 peptides (Figure S10 & Table S3 in the

Supporting Information). To confirm the accuracy of our microarray data (which are based on a solid-phase assay), we picked three representative peptides with different k_{obs} values (high, moderate, and low) for independent verification with a solution-phase HPLC assay (Figure 2c & Table 1). The results

Table 1: k_{obs} values obtained from the microarray and HPLC assays.[a]

Peptide	k_{obs} with array	k_{obs} with HPLC
E2: PVPPG–*p*T–PAPPG	0.13 ± 0.03	0.17
L5: HSSAA–*p*T–PNLGP	0.077 ± 0.03	0.064
E8: HSESA–*p*S–PSALS	ND[b]	ND[b]

[a] Values are given ± the standard deviation. [b] ND: not determined; the k_{obs} value was too small to be determined accurately.

confirmed that the k_{obs} values obtained from the microarray were consistent with those measured by using the HPLC assay. Furthermore, the microarray assay appeared to be quite sensitive, capable of detecting "weak" substrates (for example, **E8** in Figure 2b,c) that would have eluded the HPLC assay.

We next used the peptide microarray to generate the corresponding substrate fingerprints against five different phosphatases. All phosphatases tested showed distinct substrate specificities (or "fingerprints") against our peptide microarray (Figure 3a). Alkaline phosphatase is a "spurious"

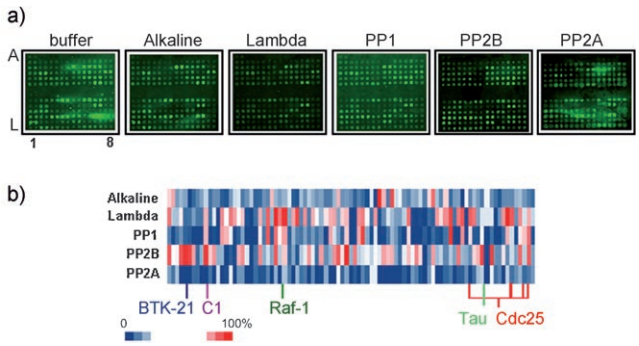

Figure 3. Substrate fingerprints of 5 different phosphatases (and buffer only as a control) against the panel of 87 peptides. The grid format of the array is the same as that shown in Figure 2a. a) Original microarray images. b) Heat map of the extracted data, with selected peptides identified. See Table S5 in the Supporting Information for details. The scale bar represents the relative dephosphorylation ratio.

enzyme known to dephosphorylate a variety of phosphate-containing molecules (including phosphopeptides/proteins).[16] Interestingly, it showed a clear preference towards *p*Ser over *p*Thr amongst the phosphopeptides (Figure S14 in the Supporting Information), which is a key criteria routinely used to distinguish acid/alkaline phosphatases from "genuine" Ser/Thr phosphatases.[16] Lambda phosphatase dephosphorylated most of the 87 peptide substrates in our microarray, which agrees well with its known broad substrate specificity.[14] Of the three mammalian Ser/Thr phosphatases, PP1, PP2B, and PP2A, only PP2A was previously shown to be involved in Pin1 regulation pathways.[12] PP1 was previously reported to show low activity against most peptide substrates.[17] Correspondingly, it weakly dephosphorylated most peptides in our microarray. PP2B, also called calcineurin, is a heterodimer made of two major subunits, namely CnA and CnB.[18] We expressed the CnA subunit (the phosphatase subunit) and tested it against our peptide microarray. The results showed that CnA dephosphorylated most peptide substrates. Of the top 6 substrates of CnA identified from our results, half of them bear a basic residue at the −3 position (BTK-21, C1, and Tau; see Table S6 in the Supporting Information). This is in good agreement with previous reports regarding the substrate specificity of PP2B.[19] PP2A, as expected, dephosphorylated many of the peptide substrates in our microarray (see below), albeit with weak activity in general. Overall, the unique fingerprints generated from our experiments should offer useful information for identifying potential physiological substrates of a target phosphatase.

Pin1 isomerizes the *p*(Ser/Thr)–Pro motif in a number of phosphoproteins (for example, Cdc25 and Tau) and, consequently, regulates their dephosphorylation with PP2A.[11,12,20] This regulatory mechanism plays an important role in a variety of cellular activities and is associated with human diseases including cancer and Alzheimer's disease.[11] PP2A is the only phosphatase identified thus far that takes part in Pin1-regulated dephosphorylation. We wondered whether our peptide microarray could be used to investigate Pin1-regulated dephosphorylation events caused by PP2A and to identify new Pin1-regulated PP2A substrates. We carried out the dephosphorylation experiment with PP2A, with or without Pin1, on the peptide microarray (Figure 4a). As shown in Figure 4b, when Pin1 regulation scores of ±0.1 were set as the cut-off thresholds, the affected peptides could be broadly classified into different groups: 1) "Expected" (>±0.1 score), where the peptides derived from previously reported Pin1-regulated protein substrates of PP2A, such as Cdc25 (T48);[12,20] 2) "unexpected" (<±0.1 score), where the peptides derived from previously known Pin1-regulated protein substrates of PP2A, including the two Tau peptides;[12,20] and 3) "new" (>±0.1 score), where the peptides derived from proteins not previously known to be Pin1-regulated PP2A substrates. These are highlighted with an asterisk in Figure 4b and include (left to right) p53, A16, p73, Bcl-2 (S87), p54nrd, cyclin D and E, Sil (S699), E6, and Sil (T574). Of this list, Bcl-2 (S87) caught our attention immediately, as it is a phosphopeptide derived from the antiapoptotic protein Bcl-2 which plays a key role in apoptosis and cancer.[21] It is known that the activity of Bcl-2 is highly regulated by phosphorylation under or oxidative stresses, and PP2A may be involved in dephosphorylation of Bcl-2.[22] Recent studies have also indicated that Pin1 can interact with Bcl-2;[23] however, the tripartite relationship has not yet been established. In our current study, the regulation experiment has provided a rapid and potentially accurate means to predict the vital interplay among these three important proteins. To confirm this, we carried out cell-based experiments to investigate the effect of Pin1 on Bcl-2 dephosphorylation by using Pin1 WT ($Pin1^{+/+}$) and Pin1 knockout ($Pin1^{-/-}$) mouse embryonic fibroblasts (MEFs; Figure 4c). Consistently, both $Pin1^{+/+}$ and $Pin1^{-/-}$ MEFs showed no difference in the level of Bcl-2 protein expression, a result

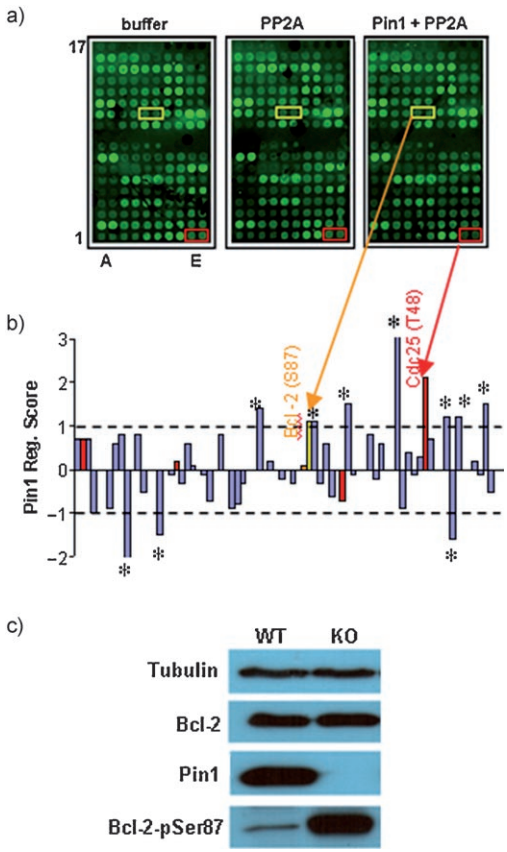

Figure 4. Pin1-regulated dephosphorylation by PP2A. a) Array images upon treatment with 1) buffer, 2) PP2A only, 3) Pin1+PP2A. The boxed spots correspond to the Bcl-2 (S87) peptide (yellow) and two of the Cdc25 peptides (red). b) Pin1 regulation score for PP2A activity against the panel of 76 peptides. Selected peptides are highlighted by colors or with asterisks. c) Cell-based experiments with $Pin1^{+/+}$ (wild-type, WT) and $Pin^{-/-}$ (knockout, KO) cell lines to confirm the up regulation of Bcl-2 (S87) protein dephosphorylation by PP2A in the presence of Pin1. PP2A is endogenously expressed in both cell lines.

suggesting that Pin1 did not affect the expression of Bcl-2. Importantly, we observed a dramatic increase in the phospho-Bcl2 (Ser87) protein level in $Pin1^{-/-}$ MEFs. These results suggest that in the absence of Pin1, PP2A probably displayed significantly reduced phosphatase activity, thereby resulting in an accumulation of phospho-Bcl2 (S87) protein. At this moment, we can not rule out the possible involvement of other Ser/Thr phosphatases (rather than PP2A) in regulating Bcl-2 dephosphorylation in $Pin1^{-/-}$ MEFs.

In conclusion, we have developed a peptide microarray capable of high-throughput determination of Ser/Thr phosphatase activities. The strategy should be amenable for future studies of other phosphatases. By determining the dephosphorylation of putative peptide substrates and generating the so-called substrate fingerprint, we were able to delineate the substrate preference of the enzyme in vitro and in the presence of regulating proteins. We showed that the strategy could be used to discover new phosphatase biology. This was illustrated with the example of PP2A, a previously known Pin1-regulated enzyme; from initial microarray findings, we were able to validate, in a cell-based assay, the suggestion that Bcl-2 dephosphorylation is indeed dependent on Pin1 expression. This might provide useful information for the future discovery of potential Pin1 inhibitors. Since our strategy utilizes purified enzymes, it might not work well with phosphatases that are active only in multimeric complexes. Additionally, since at most only 1000–10000 peptides could be accommodated on each glass slide, our platform can only cover a small fraction of all the possible sequences of a target phosphatase. Nevertheless, this method should provide a useful chemical tool in the emerging field of catalomics.[24]

Received: August 1, 2007
Revised: November 9, 2007
Published online: January 18, 2008

Keywords: microarrays · peptides · phosphatases · phosphorylation · substrate specificity

[1] G. Manning, D. B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, *Science* **2002**, *298*, 1912–1934.
[2] T. Pawson, J. D. Scott, *Science* **1997**, *278*, 2075–2080.
[3] L. Bialy, H. Waldmann, *Angew. Chem.* **2005**, *117*, 3880–3906; *Angew. Chem. Int. Ed.* **2005**, *44*, 3814–3839.
[4] a) J. Cho, R. Krishnaraj, M. Itoh, E. Kitas, W. Bannwarth, H. Saito, C. T. Walsh, *Protein Sci.* **1993**, *2*, 977–984; b) Z. Y. Zhang, D. Maclean, D. J. McNamara, T. K. Sawyer, J. E. Dixon, *Biochemistry* **1994**, *33*, 2285–2290; c) U. Dechert, M. Affolter, K. W. Harder, J. Matthews, P. Owen, I. Clark-Lewis, M. L. Thomas, R. Aebersold, F. R. Jirik, *Eur. J. Biochem.* **1995**, *231*, 673–681.
[5] a) M. Garaud, D. Pei, *J. Am. Chem. Soc.* **2007**, *129*, 5366–5367; b) M. C. Pellegrini, H. Liang, S. Mandiyan, K. Wang, A. Yuryev, I. Vlattas, T. Sytwu, Y.-C. Li, L. P. Wennogle, *Biochemistry* **1998**, *37*, 15598–15606; c) S. Walchli, X. Espanel, A. Harrenga, M. Rossi, G. Cesareni, R. Hooft van Huijsduiinen, *J. Biol. Chem.* **2003**, *279*, 311–318; d) S. W. Vetter, Y.-F. Keng, D. S. Lawrence, Z.-Y. Zhang, *J. Biol. Chem.* **2000**, *275*, 2265–2268; e) P. Wang, H. Fu, D. F. Snavley, M. A. Freitas, D. Pei, *Biochemistry* **2002**, *41*, 6202–6210; f) X. Espanel, S. Wälchli, T. Rückle, A. Harrenga, M. Huguenin-Reggiani, R. Hooft van Huijsduijnen, *J. Biol. Chem.* **2003**, *278*, 15162–15167; g) X. Espanel, R. Hooft van Huijsduijnen, *Methods* **2005**, *35*, 64–72.
[6] M. Köhn, M. Gutierrez-Rodriguez, P. Jonkheijm, S. Wetzel, R. Wacker, H. Schroeder, H. Prinz, C. M. Niemeyer, R. Breinbauer, S. E. Szedlacsek, H. Waldmann, *Angew. Chem.* **2007**, *119*, 7844–7847; *Angew. Chem. Int. Ed.* **2007**, *46*, 7700–7703.
[7] To avoid confusion, we use the terms "membrane-based microarray" and "peptide microarray" throughout the manuscript to distinguish microarray strategies carried out on membranes and on glass slides, respectively.
[8] P. R. Kumaresan, K. S. Lam, *Mol. Biosyst.* **2006**, *2*, 259–270.
[9] a) B. T. Houseman, J. H. Huh, S. J. Kron, M. Mrksich, *Nat. Biotechnol.* **2002**, *20*, 270–274; b) J. R. Falsey, M. Renil, S. Park, S. Li, K. S. Lam, *Bioconjugate Chem.* **2001**, *12*, 346–353; c) M. L. Lesaicherre, M. Uttamchandani, G. Y. Chen, S. Q. Yao, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2085–2088; d) C. M. Salisbury, D. J. Maly, J. A. Ellman, *J. Am. Chem. Soc.* **2002**, *124*, 14868–14870.
[10] U. Reineke, R. Volkmer-Engert, J. Schneider-Mergener, *Curr. Opin. Biotechnol.* **2001**, *12*, 59–64.
[11] a) K. P. Lu, Y. C. Liou, X. Z. Zhou, *Trends Cell Biol.* **2002**, *12*, 164–172; b) Y. C. Liou, A. Sun, A. Ryo, X. Z. Zhou, Z. X. Yu, H. K. Huang, T. Uchida, R. Bronson, G. Bing, X. Li, T. Hunter, K. P. Lu, *Nature* **2003**, *424*, 556–561; c) A. Ryo, M. Nakamura, G. Wulf, Y. C. Liou, K. P. Lu, *Nat. Cell Biol.* **2001**, *3*, 793–801.

[12] X. Z. Zhou, O. Kops, A. Werner, P. J. Lu, M. Shen, G. Stoller, G. Kullertz, M. Stark, G. Fischer, K. P. Lu, *Mol. Cell* **2000**, *6*, 873 – 883.
[13] K. Martin, T. H. Steinberg, L. A. Cooley, K. R. Gee, J. M. Beechem, W. F. Patton, *Proteomics* **2003**, *3*, 1244 – 1255.
[14] S. Zhuo, J. C. Clemens, D. J. Hakes, D. Barford, J. E. Dixon, *J. Biol. Chem.* **1993**, *268*, 17754 – 17761.
[15] A. R. Fersht, *Enzyme Structure and Mechanism*, 3rd ed., Freeman, New York, **1985**, pp. 105 – 106.
[16] L. A. Pinna, A. Donella-Deana, *Biochim. Biophys. Acta, Mol. Cell Res.* **1994**, *1222*, 415 – 431.
[17] M. Bollen, *Trends Biochem. Sci.* **2001**, *26*, 426 – 431.
[18] J. Aramburu, J. Heitman, G. R. Crabtree, *EMBO Rep.* **2004**, *5*, 343 – 348.
[19] A. Donella-Deana, M. H. Krinks, M. Ruzzene, C. Klee, L. A. Pinna, *Eur. J. Biochem.* **1994**, *219*, 109 – 117.
[20] J. Jordens, V. Janssens, S. Longin, I. Stevens, E. Martens, G. Bultynck, Y. Engelborghs, E. Lescrinier, E. Waelkens, J. Goris, C. Van Hoof, *J. Biol. Chem.* **2006**, *281*, 6349 – 6357.
[21] S. W. Fesik, *Nat. Rev. Cancer* **2005**, *5*, 876 – 885.
[22] S. S. Lin, M. C. Bassik, H. Suh, M. Nishino, J. D. Arroyo, W. C. Hahn, S. J. Korsmeyer, T. M. Roberts, *J. Biol. Chem.* **2006**, *281*, 23003 – 23012.
[23] N. Pathan, C. Aime-Sempe, S. Kitada, S. Haldar, J. C. Reed, *Neoplasia* **2001**, *3*, 550 – 559.
[24] H. Sun, S. Chattopadhaya, J. Wang, S. Q. Yao, *Anal. Bioanal. Chem.* **2006**, *386*, 416 – 426.

