Biosensors and Bioelectronics 24 (2009) 2871–2877

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Development of a universal phosphorylated peptide-binding protein for simultaneous assay of kinases

Wei Li[a,b,d,1], Lijun Bi[b,1], Wenhua Wang[b,d,1], Yongjin Li[a], Yafeng Zhou[a], Hongping Wei[a], Tao Jiang[b], Lin Bai[b,d], Yuanyuan Chen[b], Zhiping Zhang[a], Xinghua Yuan[c], Jianping Xiao[c], Xian-En Zhang[a,*]

[a] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China
[b] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[c] Cancer hospital, Chinese Academy of Medical Sciences, Beijing 100210, China
[d] Graduate School, Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 21 November 2008
Received in revised form 18 February 2009
Accepted 18 February 2009
Available online 3 March 2009

Keywords:
Protein kinase assay
Alkaline phosphatase
Phosphorylated peptide-binding protein
Peptide chip

ABSTRACT

This study describes the development of a universal phosphorylated peptide-binding protein designed to simultaneously detect serine, threonine and tyrosine kinases. The *Escherichia coli* alkaline phosphatase (EAP) is a well-defined nonspecific phosphated monoesterase and Ser-, Thr- or Tyr-phosphorylated peptides served as substrates for EAP in preliminary experiments. Based on the known catalytic mechanism of EAP, the recombinant site-directed mutant EAP-S102L was generated, whose catalytic activity was blocked, but its binding ability was preserved. For EAP-S102L the catalytic rate constant, k_{cat}, was reduced by a factor of 1000, while the Michaelis–Menten constant, K_m, remained almost unchanged. Crystallographic analysis of the EAP-S102L/phophorylated peptide complex revealed that EAP-S102L could bind the phosphate group of the phosphorylated peptide but lacked nucleophilic attack potential which was essential for the catalytic ability of EAP. Finally, by combining the fluorescence-labeled EAP-S102L with non-phophorylated peptide chips, kinases could be detected from tumor cell samples. The recombinant EAP-S102L construct is perhaps the first functional binding protein derived from a native enzyme, illustrating how one single mutation tremendously alters protein function.

1. Introduction

Protein kinases are known to regulate the majority of cellular pathways, especially those involved in signal transduction (Hubbard and Till, 2000). Perturbation of protein kinase-mediated cell signaling pathways is a frequent cause of disease, particularly cancers, diabetes and inflammation (Bialik and Kimchi, 2006). Although hundreds of protein kinases have been reported to date (Kato et al., 2008), the number and type of kinases expressed by cells and tissues under varying situations is far from being identified. Therefore protein kinase assay is of both biological and clinical importance.

To profile kinases or measure the kinase activity, various methods have been developed (Minor, 2003; Rininsland et al., 2005; Kim et al., 2007). In general, they may be antibody based or non-antibody based. Most of the methods use short peptides as the specific substrates of the target kinases. After phosphorylation by the kinases, the phosphorylated peptides are recognized and bound by the specific antibodies that are fluorescence or enzyme-labeled. However, considering that there are hundreds of kinases in the human body and each of them has a specific substrate, the generation of multiple monoclonal (mAbs) for high throughput identification of various kinases is neither feasible nor cost effective. Kazuki et al. (Inamori et al., 2005) recently reported a method that allowed for the parallel analysis of multiple protein kinases using a synthetic phosphate capture molecule (*i.e.*, biotinylated zinc [II] complex). The work is an important first step towards analyzing multiple protein kinases simultaneously. Nevertheless, at present the most reliable means for simultaneous assay of multiple kinases are ATP-based protocols, which measure either the chemical labeled ATP incorporated into peptides (Green and Pflum, 2007) or ADP produced from ATP after phosphorylation reaction by kinases (Gaudet et al., 2003). The methods sometimes are named universal protein kinase assay and some of them were used in commercial available test kits already (e.g. ATP quantitative kit Kinase-Glo™, Promega, Madison, U.S.A.; ADP quantitative kit Adapta™, Invitrogen, CA, USA and ADP Quest™, DiscoveRx, CA. USA). Whereas, one worry is that the intrinsic ATPase in the reference sample introduces contaminants and therefore interferes

* Corresponding author at: Wuhan Institute of Virology, Chinese Academy of Sciences, 44 Xiaohongshan, Wuchang District, Wuhan 430071, PR China.
Tel.: +86 27 87199492; fax: +86 27 87199492.
E-mail address: x.zhang@wh.iov.cn (X.-E. Zhang).
[1] The authors contributed equally to this work.

0956-5663/$ – see front matter.
doi:10.1016/j.bios.2009.02.020

with ATP incorporation. Another promising mean to profile kinase activities is the MALDI MS method of Mrksich's laboratory (Min et al., 2004). The method is powerful but needs expensive instrument and multistep sample treatment. So, there is still a great need for building a new assay system towards easy to use and low cost but with comparable selectivity and sensitivity.

This study aimed to assay various O-phosphate-type protein kinases (Ser, Thr and Tyr, which constitute 95% of protein kinases in eukaryotic cells) (Arena et al., 2005) using a phosphorylated peptide-binding protein. This was accomplished by generating a recombinant derivative of the *E. coli* alkaline phosphatase (EAP). EAP is a nonspecific phosphate monoesterase that can bind and hydrolyze phosphate monoesters universally (Llinas et al., 2005). Since the catalytic mechanism of EAP was established (Coleman, 1992) we were able to design a protein that retained binding activity but was deficient in its ability to hydrolyze substrate phosphate groups. By combining the fluorescence-labeled binding protein with the non-phosphorylated peptide chip different kinases could be distinguished simultaneously.

Here, cyclin-dependent kinase 1 (CDK1, serine kinase), EGF-receptor tyrosine kinase (EGFR, threonine kinase) and Glycogen synthase kinase 3β (GSK3β, tyrosine kinase) were used to phosphorylate three O-phosphate-type peptides. The site-directed mutant EAP-S102L was constructed and kinetic and binding affinity analysis to phosphorylated peptides was characterized by stopped-flow and surface plasmon resonance (SPR), respectively. Crystallographic analysis of the EAP-S102L/phosphorylated peptide complex crystal provided direct evidence of EAP-S102L specificity binding phosphate group. Simultaneous assays of three types of kinases, CDK1, EGFR and GSK3β in cell culture and cancer tissues, respectively, were successfully carried out by combining the use of the Cy3-labeled S102L, non-phosphorylated peptide chips and fluorescence scanning.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli DH5α was used for all bacterial transformations and plasmid propagations and strain SM547 was used for protein expression. Plasmids pEK48 and pASK75 (kindly provided by Prof. Kantrowitz (Boston College) and Prof. Cass (Imperial College)) were used to construct the vectors for expression of wild type and mutant EAP. *pASK75-EAP-His* expressing the fusion EAP/His-tag protein was constructed. All cloning procedures were confirmed by DNA sequencing.

2.2. Expression and purification of the proteins

E. coli SM547 cells transformed with the plasmid pEK48 and pASK75 was used as the host strain for expression of wild type and mutant EAP. To start the growth, 5 mL overnight cultures were prepared from these transformations in LB with ampicillin (200 μg/mL), and incubated at 37 °C with shaking. 1 L LB media with the same antibiotics was inoculated with 5 mL overnight culture and then induced with 0.2 μg/mL tetracycline and continuously cultured overnight at 26 °C. Periplasmic extracts were prepared using a lysozyme protocol (Pierce et al., 1997). Periplasmic extracts were centrifuged at 8000 × *g*, 30 min and aliquots were then applied to resource-Q ion-exchange chromatography (Amshambioscience, Sweden) and was eluted with a NaCl gradient from 0% to 40% in TMZP buffer (0.01 M Tris–HCl, 0.001 M $MgCl_2$, 10^{-5} M $ZnSO_4$, 10^{-4} M NaH_2PO_4, pH 7.4). The mutant EAP was purified by size-exclusion chromatography on a Superdex-200 column (Amshambioscience) eluted with the buffer 0.01 M Tris–HCl, 0.15 M NaCl, pH 7.4 for crystal growth. The purified protein was assessed at each step by SDS-PAGE and finally stored at −80 °C.

2.3. Enzyme kinetic assays

Steady-state enzyme kinetic assays were documented spectrophotometrically using *p*-nitrophenyl phosphate as substrate. The release of the *p*-nitrophenolate chromophore product was monitored by measuring the absorbance at 410 nm. Temperature was regulated at 25.0 ± 0.5 °C using a circulating constant-temperature bath. The buffer system was 1.0 M Tris pH 8.0.

2.4. SPR experiments

Interactions of EAP and EAP-S102L with phosphorylated peptides were carried out on a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden). The BIAcore 3000 system contains a dual-channel measuring cell. The carboxymethylated dextran surface-modified chip (CM5 chip) was employed to prepare the S102L sensor chip according to the amine-coupling protocol of the BIAcore manual. Before measurement, the sensor chips were equilibrated with running buffer at the rate of 20 μL/min. The running buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 0.01 mM $ZnCl_2$ and 0.005% [v/v] Tween-20) was filtered through Millipore Film (pore size 0.22 mm) and degassed before use. Samples were injected at different concentrations at a flow rate of 20 μL/min for 2 or 3 min. The curves were fitted to a 1:1 Langmuir binding model (BIAevaluation 4.1 software) to obtain the equilibrium and kinetic constants.

2.5. Crystallization

The vapor diffusion method was used to crystallize EAP-S102L using the hanging drop method by mixing 1 μL protein solution and 1 μL reservoir solution. Before use, EAP-S102L (25–30 mg/mL) was dialyzed against a buffer composed of 20% saturated $(NH_4)_2SO_4$, 100 mM Tris pH 9.5. Crystals formed in about one week at 16 °C in reservoirs where the ammonium sulfate was between 39% and 43% in the presence of 100 mM Tris pH 9.5 (Tibbitts et al., 1994). Crystals are improved by microseeding after an 8–10 h incubation. The phosphorylated peptide P1-P was dissolved in reservoir solution at the concentration of 10 mg/mL. Then one hanging drop was introduced to 2 μL peptide solution to soak the crystals for 4 min prior to data collection.

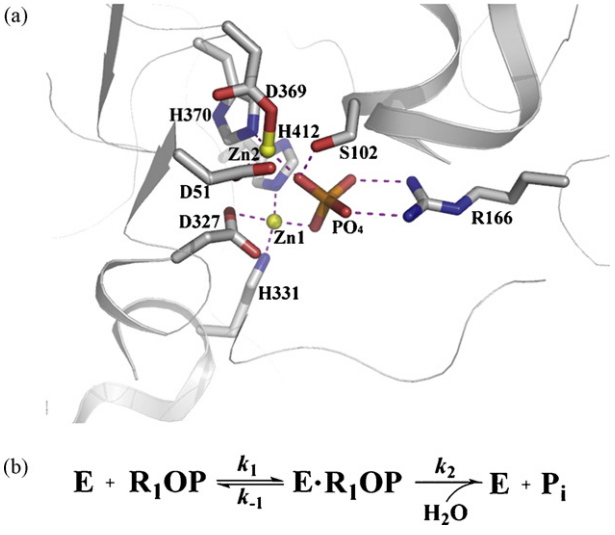

Fig. 1. Catalytic center of EAP (a) and hydrolytic reaction formula for EAP (b).

Table 1
The kinetic constants for EAP and EAP-S102L[a].

Enzyme	K_{cat} (S^{-1})	K_m (μM)	K_{cat}/K_m ($M^{-1}S^{-1}$)	Specific activity (U/mg)
WT	11.81 ± 0.32	20.62 ± 3.31	5.72×10^5	12
S102L	$(9.4 \pm 0.28) \times 10^{-3}$	78.85 ± 2.5	1.19×10^2	8.2×10^{-3}

[a] Substrate: *p*-nitrophenyl phosphate.

2.6. Cy3 labeling of EAP-S102L

EAP-S102L was labeled by Cy3 biofunctional dye by Cy3 Fluor Protein Labeling Kit (Invitrogen, Molecular Probes). Unconjugated Cy3 was removed by gel filtration chromatography as described (Christoph et al., 2001).

2.7. In vitro kinase assays

The kinase activity of GSK3β, EGFR and CDK (which are Ser, Thr and Tyr protein kinases respectively) was determined by assaying their respective phospho-products generated during the *in vitro* kinase reaction experiments using SPR and peptide chips. The *in vitro* CDK1 reaction mixture contained 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM Mg^{2+}, 1 mM ATP, and 300 μM of P1 peptide. The composition of the EGFR *in vitro* reaction system was the same as CDK1's except for the corresponding peptide substrate P2 and the kinases. The peptide substrate of GSK3β is P3 and the additional 1.25 mM $MnCl_2$ was needed for this reaction system in contrast with the other kinases. The negative and positive controls were set up for every protein kinase reaction. The negative control reactions were carried out in the absence of kinase and the substrate peptides were substituted by the corresponding phospho-peptide in the positive control reaction systems.

2.8. Preparation of the peptide chip

The peptide array was arranged on the streptavidin-modified glass chip (purchased from Xenoprobe Company, USA). Proper peptide orientation on the chip was carried out by immobilizing the peptides to the chip via biotin/streptavidin interactions. On each spot, 50 nL of diluted biotinylated-peptide solution at a concentration of 300 μM was added using the pin tool (V&P scientific Inc. USA). The chip was then incubated at room temperature for 0.5 h. After removal of the solution, the spots were blocked with PBS blocking solution containing 5% BSA. The spots were washed three times using assay buffer and protein kinase activity measured (Houseman et al., 2002).

2.9. Cell culture and clinical tissue lysate

Tumor cells (HeLa, HEK293 and A431) were cultured in 10% FBS DMEM and washed with cold PBS three times and then lysed using cold RIPA (PBS, 150 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, 2 μg/mL Aprotinin and 2 μg/mL Leupeptin).

The clinical tissues examined were obtained from the Beijing Cancer Hospital. Sample 1, colorectal cancer, 53 years old, female; sample 2, colorectal cancer, 44 years old, male; sample 3, colorectal cancer, 67 years old, male were frozen in liquid N_2 for grinding and then thawed in the presence of the RIPA buffer. Samples were centrifuged at 10k × *g*, 4 °C, for 30 min and the supernatants used in Western blot and peptide array assays.

2.10. Detection of protein kinase on the peptide chip

After the protein kinase incubation period was over, the reaction buffer was removed and the chip was blocked with 5% BSA PBS for 20 min, washed three times with PBS and 2 mg/mL of labeled Cy3-labled EAP-S102L was added to the wells and incubated at 4 °C for 30 min for phosphorylated peptide binding. Unbound Cy3-EAP-S102L was removed and the phosphorylated peptide-bound Cy3-EAP-S102L was detected on the peptide chip using the GenePix 4000B (Axon Instrument) fluorescence Scanner with the sensitivity of 0.1 fluorophores/μm^2 and the data was analyzed with GenePix 4.0 analysis software (Axon Instruments).

2.11. Western blot analysis

The cell culture and clinical tissue lysate were separated by electrophoresis through SDS-PAGE, and electrotransfered to Polyvinylidene-Fluoride (PVDF) membrane. The second antibody conjugated with horseradish peroxidase was used, and positive immunoreactive bands were detected by 3,3′,5,5′-tetramethylbenzidine (TMB).

3. Results

3.1. Enzymatic peptide substrates of EAP

Protein kinase specificities and activities were defined using synthetic peptides since the kinase-specific determinants were located in short, primary sequence regions surrounding phosphorylation sites (Kemp and Pearson, 1990). In this study, three synthetic peptides, P1 (H-A-T-P-P-K-K-E-A-D), P2 (A-D-E-Y-L-I-P-Q-Q), and P3 (G-P-H-R-S-T-P-E-S-R-A-A-V), serving as the substrates for CDK1, EGFR and GSK3β, respectively, were used for analysis of kinetic and binding-affinity of EAP. EAP was incubated with the synthetic

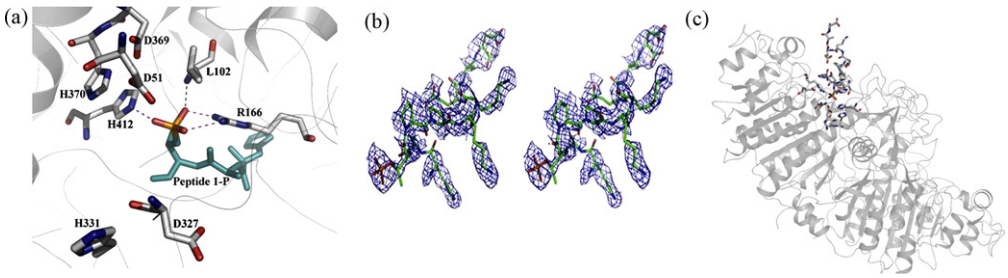

Fig. 2. Structural analysis of S102L-P1-P. (a) Stick representation of the hydrogen bonds between the P1-P phosphate moiety and residues in the binding pocket of S102L involving Arg-166, Leu-102 and His-412. (b) Stereoview of P1-P with *2Fo-Fc* density maps of the phosphorous moiety (1.0σ) and of the other part (0.8σ). (c) Ribbon and stick representation of the overall structure.

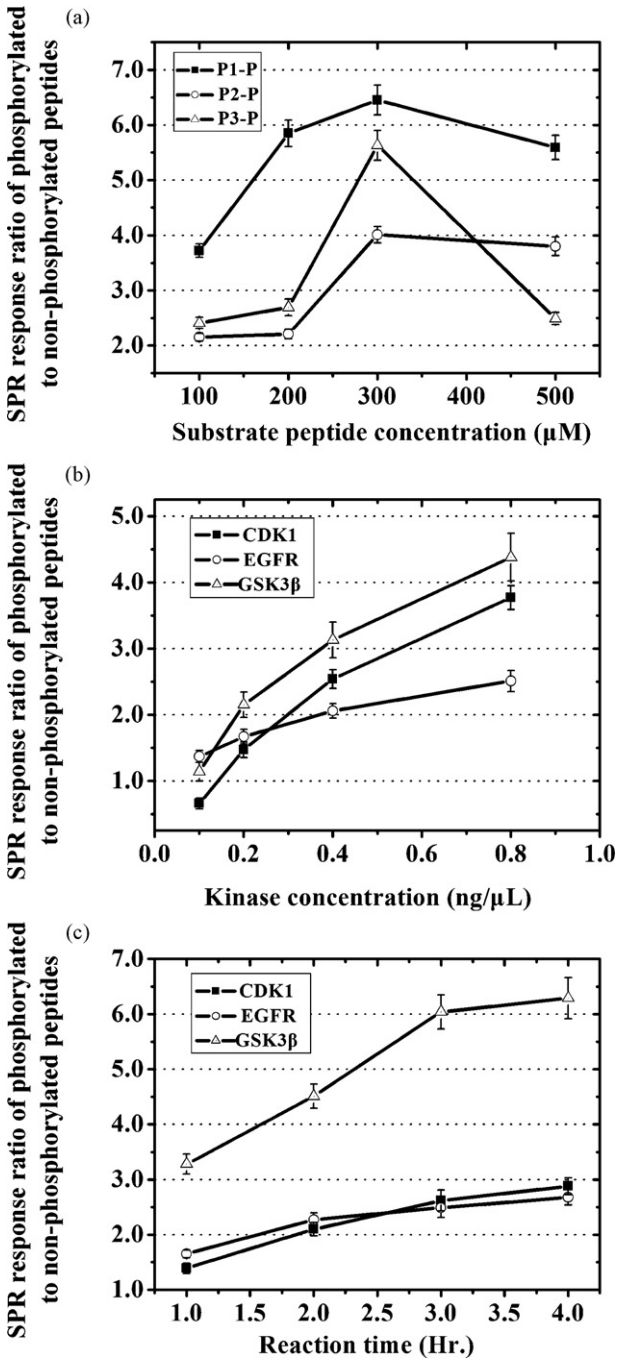

Fig. 3. Optimization of the kinase reaction conditions. (a) The response of the SPR chip to the phosphorylated peptides at different concentrations. At each concentration tested, non-phosphorylated substrate peptides with the same concentration were used to define the background levels. (b) The responses of the SPR chip to the kinase reaction solutions at different reaction times. (c) The responses of the SPR chip to the kinase reaction solutions using different kinase concentrations.

peptides (P1, P2, P3) and their phophorylated products (P1-P, P2-P, P3-P), respectively, and the reaction mixtures were analyzed by mass spectrometry. As shown in Supplemental Figure S1, the molecular weight of P1-P was 1173.7 Da. After incubation with EAP, the P1-P peak was replaced with a 1093.6 Da peak identical to the molecular weight of P1, indicating that P1-P had been converted to P1. Similar results were observed when incubating EAP with P2-P or P3-P, respectively (data not shown), suggesting that like other phosphate monoesters, the phophorylated peptides could also serve as EAP substrates.

3.2. Construction and analysis of EAP-S102L

The crystal structure of EAP was resolved (Coleman, 1992) and details of catalytic center are shown in (Fig. 1a). An earlier study demonstrated that EAP-mediated catalysis involved two sequential nucleophilic attacks on the in-line phosphate groups (Wojciechowski and Kantrowitz, 2002). The first nucleophilic attack on the phosphate by the hydroxyl group of Ser-102 resulted in a phosphomonoester (E-P). Upon formation of the covalent E-P intermediate, the phosphate group moved in close proximity to the active site cavity thereby maintaining interactions with both Arg-166 and the zinc ions. Subsequently Zn1 attacked the covalent E-P intermediate forming the non-covalent E Pi complex. Finally, the magnesium-dependent EAP release of Pi completed the catalysis reaction (Fig. 1b).

We hypothesized that if Ser-102 were replaced by Leu-102 (a nonpolar residue without a hydroxyl group) it would prevent EAP from forming the phosphomonoester bond with the phosphorous group and therefore be unable to carry out catalysis but would still bind (but not catalyze) via the Arg-166 which forms the hydrogen bond with the phosphate group. To this end, the EAP-S102L site-directed mutant was constructed, purified and subjected to enzyme kinetic analyses using stop-flow technology. It was demonstrated that enzymatic activity was reduced by 1000-fold but binding remained indistinguishable from wild-type EAP (Table 1).

3.3. SPR analysis

The EAP-S102L-modified CM5 chip was used in SPR experiments and the P1, P2, P3 and P1-P, P2-P, P3-P peptides were examined individually. The response unit of EAP-S102L binding to the phosphorylated peptides was about two-fold higher than that of non-phosphorylated peptides regardless of the concentration tested (100 or 300 μM). SPR results indicated that EAP-S102L could distinguish phosphorylated from unphosphorylated peptides.

As shown in Supplemental Table S1, the response unit signals were proportional to the sample concentrations at certain ranges. The sensorgrams were fit to a 1:1 Langmuir binding model (BIA evaluation 4.1 software) to obtain kinetic constants. Dissociation constants (K_D values) of EAP-S102L were determined to be 44, 40.8 and 38.2 μM for P1-P, P2-P and P3-P respectively.

3.4. Crystallographic analysis of the phosphorylated peptide complex

To understand the molecular interactions between EAP and phosphorylated peptides the crystal structure of EAP-S102L complexed with phosphorylated peptide P1-P was determined at 2.3 Å and the structure was solved by molecular replacement using the 1B8J (PDB id) search model as a starting model. The final complex structure was refined to the $R_{working}/R_{free}$ of 0.234/0.271. Data processing and refinement statistics are shown in Supplemental Table S2. The overall structure was remarkably similar to the wild-type structure except that a loop instead of an α-helix spanned residues 325–331 following the removal of Zn^{2+} and Mg^{2+} during crystallization, which is coincidence with the previous observation (Sowadski et al., 1983) (Fig. 2a). Two phosphorylated peptide P1-Ps bound to monomer A and B, respectively, was easily discernable in all model structures (Fig. 2b). As the electron density of P1-P in monomer A is better than that in B, the following discussion is based on the P1-P bound to monomer A.

Most contact points between EAP and P1-P were restricted to the P1-P phosphate moiety. This phosphate moiety was stabilized by hydrogen bonds as shown in Fig. 2a. The distances between phosphorus oxygen O3 and NH1 of Arg166, O2 and NH2 of Arg166 are 2.5 Å and 3.2 Å, respectively. Previous studies also showed that

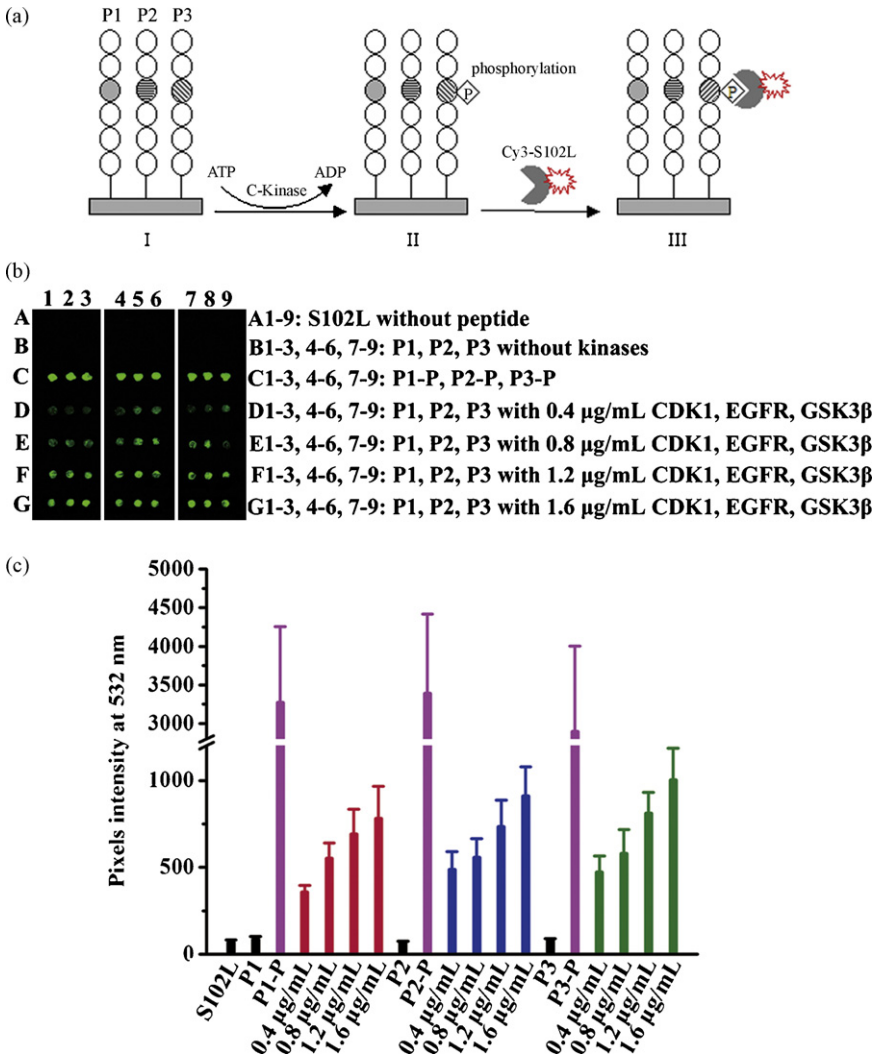

Fig. 4. (a) Peptide chip schematic. (I) Peptide chip where the peptide array was deposited. (II) Site-specific phosphorylation of peptide P1, P2, P3 at specific sites using C-kinase. (III) Preferential binding of Cy3-labeled S102L to the phosphorylated peptides resulting in a fluorescence signal at respective positions on the C-peptide chip. (b, c) Commercial kinase evaluations. Analysis of CDK1, EGFR and GSK3β (0.4, 0.8, 1.2, 1.6 μg/mL) using the peptide chip. (b) A typical fluorescence image obtained. (c) Quantitative analysis of the fluorescence signals (SE, $n = 3$).

Arg-166 binding utilized these bonds (Kim and Wyckoff, 1991). The shift of the Leu-102 side chain away from the phosphorus moiety resulted in the formation of a hydrogen bond between O3P and the nitrogen backbone of Leu-102 (which was 2.8 Å apart) that likely resulted in the low catalytic activity of EAP-S102L. The phosphorus oxygen O1P was also in a position to form a hydrogen bond with NE2 of His-412 whose distance was 3.0 Å. These interactions explained the high affinity between EAP-S102L and the phosphorylated peptide compared to the non-phosphorylated peptide.

In addition, structure analysis indicated that there was a lack of specific contact points between EAP and any other part of P1-P because the active pocket could only accommodate a single phosphate moiety (Fig. 2c).

3.5. Assay optimization and analysis of commercial protein kinases

SPR assay conditions were optimized by assaying various concentrations of the kinase, peptides and varying the reaction times using an EAP-S102L-modified chip. Phosphorylation of the peptides was performed by incubating peptides in the presence of the kinase in 0.5 mL microfuge tubes. At 2 h if not specially described the reactions were subjected to SPR analysis (Fig. 3). This analysis revealed that the most efficient reaction took place using 300 μM peptides incubated for 2 h. 0.4 μg/mL CDK1 or EGFR and 0.2 μg/mL GSK3β produced a SPR response ratio of phosphorylated to non-phosphorylated peptides above 2.0.

The peptide chips (P1, P2 and P3) were used for the CDK1, EGFR and GSK3β assays (Fig. 4a). Kinases were added onto the corresponding peptide chips and enzymes were used at 0.4 μg/mL for CDK1 and EGFR and 0.2 μg/mL for GSK3β. The reactions (including positive and negative controls) were carried out for 2 h and after phosphorylation, the chips were washed with buffer before the binding reaction was carried out by adding Cy3-labeled EAP-S102L. The chips were then subjected to fluorescence scanning and the detected fluorescent signals indicated that Cy3-labeled EAP-S102L had bound to the phosphorylated, peptide-bound chips. Fig. 4b and c show that the fluorescence intensity of the positive control (phosphorylated peptides) was much greater than that of the negative control (non-phosphorylated peptides) and that the three commercial kinases (CDK1, EGFR and GSK3β) successfully phosphorylated their chip-bound substrates that were then identified by Cy3-labeled EAP-S102L. The kinase dose-dependent fluorescence intensities of the enzymatically phosphorylated sites on the chips

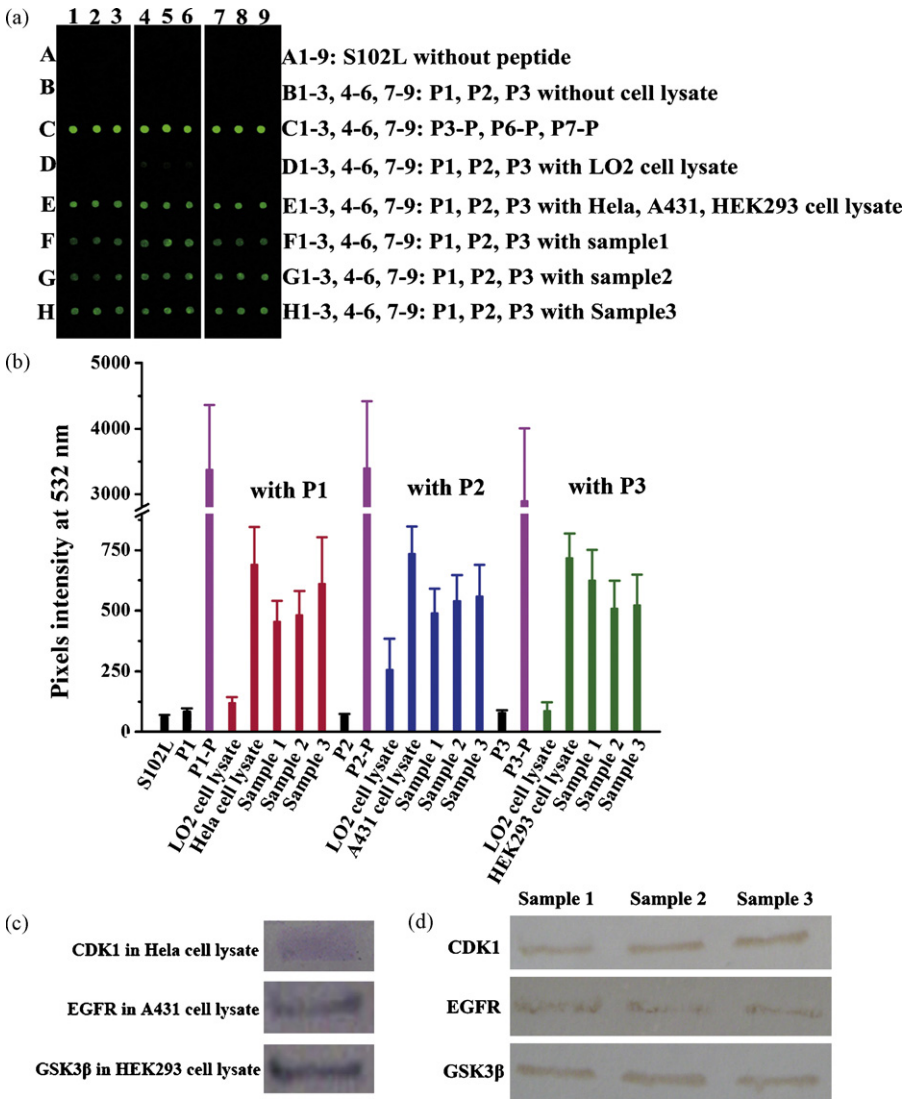

Fig. 5. Kinase evaluation in clinical samples. (a) A typical fluorescence image obtained. (b) Quantitative analysis of the fluorescence signals (SE, $n = 3$). (c, d) Western blot analysis of kinases in cell culture and cancer tissues. The human fetal hepatocyte cell line LO2 was used as an additional negative control.

were higher than that of the negative control demonstrating that this reaction could be quantified.

3.6. In vitro analysis of protein kinases activity of tumor cells and clinical samples

The A431 and HEK293 tumor cell lines known for their elevated EGFR and GSK3β levels, respectively (Yarden and Schlessinger, 1987; Kim et al., 2003), were used for protein kinase evaluations using both non-phosphorylated and phosphorylated chip-bound peptides (as the negative and positive controls, respectively) that were subsequently combined with EAP-S102L. The fluorescence intensities of the spots treated with the cell lysates were at least twice as intense as the negative control signals (Fig. 5a and b) and these results were verified by Western blot analysis (Fig. 5c).

In a similar fashion human colorectal cancer tissue samples were analyzed. The healthy control LO2 cell line that possessed low levels of each of the three kinases was used as negative control. Chip assays showed that all three protein kinases (CDK1, EGFR and GSK3β) could be identified in cancer tissue samples harvested from three different patients when compared to the LO2 negative control (signal/noise ≥2) (Fig. 5a and b) and confirmed by Western blot analysis (Fig. 5d).

4. Discussion

In this study, EAP-S102L was constructed successfully for phosphorylated peptide-binding analyses. The methodology presented in this report offers several advantages over the antibody method currently used for the detection of protein kinases. First, EAP-S102L had the potential of recognizing and binding to various phophorylated peptides since EAP is a known nonspecific phosphate monoesterase and we had approved it can hydrolyze three types of phosphorylated peptides. EAP-S102L was designed to be a "universal" O-phosphate-type phophorylated peptide-binding protein with the potential of simultaneously detecting hundreds of different protein kinases. Second, the generation of large quantities of recombinant EAP-S102L could be produced at very little cost in contrast to mAb production. Third, to obtain Cy3-labeled EAP-S102L only one labeling and separation step was required in contrast to multiple such steps if mAbs were used.

Ideally, non-radioisotope and homogeneous methods should be selected as a high throughput measurement of kinases. Since ATP is a universal substrate for kinases, the assays based on measurement of ATP or ADP is far more simple and reliable for of kinases than the antibody method. Nevertheless, as mentioned before, interference

of endogenous ATPase in the samples may be a problem. This could be avoided by using the EAP-S102L protocol.

Since the average binding constant of EAP-S102L to various phosphorylated peptides was about 4×10^{-5} M (Supplemental Table S1) and mAbs can have binding constants in the μM to pM ranges, some concerns regarding the specificity of kinase detection by EAP-S102L could be raised, however, the reduced affinity did not affect specificity since peptides were immobilized to the detection chips at a high density (300 μM) and protein kinase phosphorylation over a 2 h period allowed for a sufficient concentration of phosphorylated peptides to bind, thereby facilitating EA1-S102L binding. Therefore, any deficiencies in kinase concentrations may be overcome by extending the incubation period or by incorporating super sensitive labeling reagents (e.g. quantum dots or nanoparticles), which may increase sensitivity by several orders of magnitude (Nam et al., 2003). The final obstacle is eliminating nonspecific binding which could also significantly affect detection limit. By far, the detection limit of the method is a few micro molars, which needs to be further improved.

To our knowledge EAP-S102L is probably the first engineered "binding protein" derivative from an enzyme. Ligand–protein interactions include non-covalent intermolecular interactions that could affect the K_D. For example the streptavidin–biotin complex K_D is 10^{-14} M (Green, 1990) compared to a K_D of 10^{-5} M for the streptavidin–streptag complex (Schmidt et al., 1996). The affinity of an enzyme to its substrate is expressed as K_m, which is normally between 10^{-2} and 10^{-5} M (Wilkinson et al., 1984). Hydrolysis of phosphate monoesterase by EAP followed simple Michaelis–Menten kinetics (Fig. 1b), where $K_m = (k_{-1} + k_2)/k_1 = (k_{-1} + k_{cat})/k_1$. If $k_{cat} \ll k_{-1}$, then $K_m \approx k_{-1}/k_1 = K_D$. The conversion of EAP to EAP-S102L resulted in a 1000-fold k_{cat} decrease and both the K_m and K_D remained at 10^{-5} M, suggesting that in this particular case EAP-S102L no longer functioned as a phosphate monoesterase but behaved as a phosphate monoester-binding protein. This raised a number of interesting questions: Do enzymes originate from binding proteins, or *vice versa*? If yes, are there transition forms? If yes, could we create other binding proteins using similar methodology? We believe these answers to be yes since EAP-S102L was generated by changing only one amino acid (S102L).

5. Conclusion

We proposed a new format of the peptide chip for simultaneous assay of protein kinases. The success of the method largely relies on the generation of a phosphorylated peptide-binding protein which was derived from EAP. The preliminary study has demonstrated that the peptide chip features universality, low cost and ease of handling. This novel protein will provide an alternative method for simultaneous assay of protein kinases and high throughput screening of kinase inhibitors.

Acknowledgements

LJ Bi was supported by the National Protein Project (No. 2006CB910902). T Jiang was supported by 863 Project (No. 2006AA02A319). Supports from Chinese Academy of Sciences (Project No. KSCX2-YW-G-017 and No. KSCX1-YW-R-63) are also acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.02.020.

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