Peptide chips for the quantitative evaluation of protein kinase activity

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Peptide chips are an emerging technology that could replace many of the bioanalytical methods currently used in drug discovery, diagnostics, and cell biology. Despite the promise of these chips, their development for quantitative assays has been limited by several factors, including a lack of well-defined surface chemistries to immobilize peptides, the heterogeneous presentation of immobilized ligands, and nonspecific adsorption of protein to the substrate. This paper describes a peptide chip that overcomes these limitations, and demonstrates its utility in activity assays of the nonreceptor tyrosine kinase c-Src. The chip was prepared by the Diels–Alder-mediated immobilization of the kinase substrate AcIYGEFKKKC-NH_2 on a self-assembled monolayer of alkanethiolates on gold. Phosphorylation of the immobilized peptides was characterized by surface plasmon resonance, fluorescence, and phosphorimaging. Three inhibitors of the enzyme were quantitatively evaluated in an array format on a single, homogeneous substrate.

The translation of conventional solution-phase assays into immobilized formats has markedly increased the rate and scope of discoveries in basic science and biotechnology. Examples include the development of DNA arrays for genome wide analysis[1,2], the preparation of protein chips for the evaluation of protein–substrate interactions[3–6], and the construction of peptide chips for the evaluation of ligand–receptor interactions and enzymatic activities[7–9]. Peptide chips have recently generated widespread interest because many enzymatic processes, including kinase[10–12] and protease[13,14] activities, can be readily studied using peptides as model substrates.

This paper describes a peptide chip for the rapid and quantitative evaluation of kinase activity. The chip is based on self-assembled monolayers that present peptide ligands, and offers three principle advantages over current array formats. First, the monolayers are inert toward nonspecific interactions with protein and radioisotopes, which eliminates the need for blocking procedures[15]. Second, all the peptide ligands on the monolayer are presented in uniform environments. This results from the structural order of the monolayer substrates and the use of a selective immobilization chemistry to prepare the chips. The homogeneous presentation of peptides permits the measurement of rate and activity data that are not obtainable with the heterogeneous protein and polymer substrates typically used in kinase assays[16,17]. Third, the monolayers are compatible with a range of detection methods, including surface plasmon resonance[18,19], fluorescence, scintillation counting, and phosphorimaging. Together, these features permit the quantitative evaluation of kinase inhibitors in an array format on a single substrate. We used the peptide chip to determine the K_i values for three inhibitors of c-Src.

Results and discussion

Preparation of substrates. Self-assembled monolayers for the immobilization of the peptides were prepared by immersing a clean gold substrate in a mixture of alkanethiols 1 and 2 (refs. 20,21; Fig. 1A). The glycol groups on the monolayer prevent nonspecific interactions with protein and radioisotopes[15], and the hydroquinone groups provide a chemical handle for the selective immobilization of ligands[20,22]. Mild oxidation of the substrate converted the hydroquinone groups to benzoquinone groups, which reacted selectively and efficiently with peptide–cyclopentadiene conjugate 3a to immobilize the peptide at a density of 10 pmol/cm^2 (Fig. 1B). This particular conjugate is an optimized substrate for c-Src kinase[23,24].

Characterization of kinase activity. To characterize the enzymatic phosphorylation of a substrate presenting tyrosine peptide 3a, we applied a solution of c-Src kinase (1 unit in 30 μL reaction buffer) and [γ-^{32}P]ATP to the monolayer and allowed the reaction to proceed for 2 hours at 37°C. The substrate was then washed and quantified by phosphorimager analysis. Substrates presenting the tyrosine-containing peptide incorporated 530 cpm per cm^2 of radiolabel, whereas monolayers presenting phenylalanine conjugate 3b or monolayers presenting no peptide incorporated only background levels of radiolabel (Fig. 2A). To demonstrate the specificity of the modification, we prepared chips presenting the peptide IYAAPKKC, a substrate of c-Abl tyrosine kinase[23], and the peptide LRRASLG, a substrate of the catalytic subunit of protein kinase A[25,26]. As expected, neither substrate was modified by c-Src kinase.

We next evaluated the enzymatic modification using surface plasmon resonance spectroscopy (SPR)[18,19]. This experiment established the compatibility of our peptide chip with this analytical technique and showed that the incorporation of phosphate onto the chip was due to the generation of phosphotyrosine. Monolayers presenting tyrosine conjugate 3a were treated with a solution containing c-Src or with an identical solution that did not contain the kinase. After incubation for 4 hours at 37°C, the substrates were mounted into the SPR flow cell. The sensorgrams in Figure 2B show that an antibody against phosphotyrosine bound only to monolayers that had been treated with the kinase. Three control experiments supported the specificity of the interaction. First, monolayers presenting phosphotyrosine

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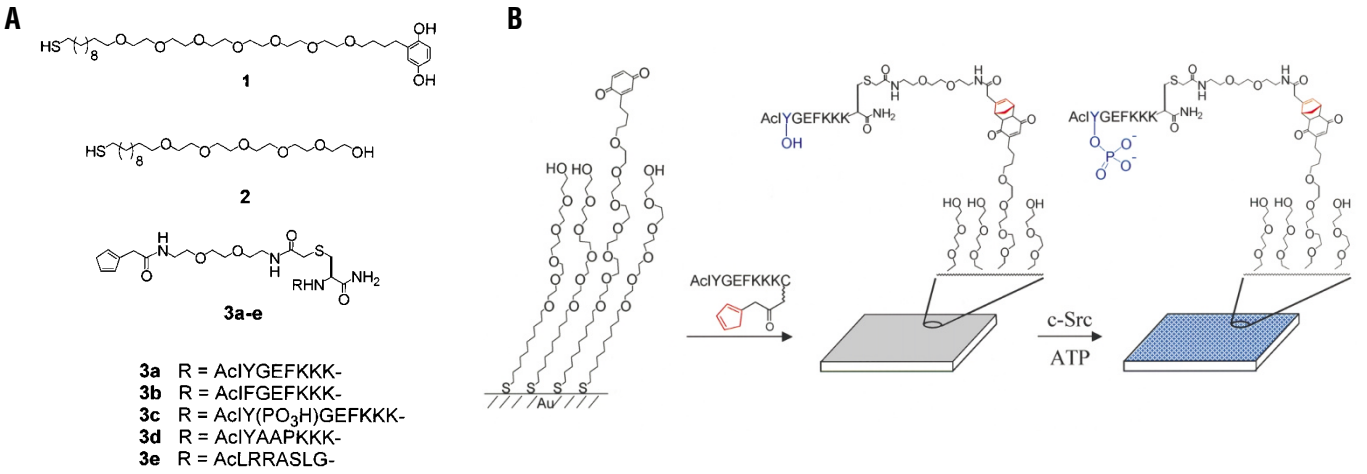

Figure 1. Peptide chip chemistry. (A) Molecules used in this work. (B) Peptide chips are prepared by applying a peptide–cyclopentadiene conjugate to a self-assembled monolayer presenting benzoquinone groups (left). The Diels–Alder reaction between the diene and quinone groups affords rapid and selective immobilization of the peptide with excellent control over ligand density. This work uses substrates presenting the peptide AcIYGEFKKKC-NH_2 (center) to characterize the on-chip phosphorylation of the peptide by c-Src kinase (right).

conjugate 3c gave a similar SPR response when treated with the antibody. Second, binding of anti-phosphotyrosine antibody to an enzymatically modified chip was completely inhibited when the antibody was preincubated with soluble phosphotyrosine (250 μM in phosphate-buffered saline (PBS)). Third, SPR experiments showed that the chips remained inert to the nonspecific adsorption of several proteins, including the "sticky" protein fibrinogen (1 mg/mL in PBS), both before and after treatment with the kinase (data not shown). Together these results confirmed the presence of phosphotyrosine on the chip and showed that phosphorylation does not compromise the inertness of the monolayers.

To demonstrate the characterization of the substrates with fluorescence microscopy, we used a commercial robot (Affymetrix 417 Arrayer, Santa Clara, CA) to array phosphotyrosine conjugate 3c onto monolayers presenting benzoquinone groups. The modified substrates were then visualized by indirect immunofluorescence. Figure 2C shows a fluorescence micrograph of five array elements together with an intensity profile of those elements. The fluorescence appeared only in regions presenting the immobilized peptide, showing that these substrates are compatible with current array technology.

Patterning multiple kinase reactions. The experiments described above suggested that the peptide chip could be used to carry out kinase assays in an array format with quantitative analysis. We therefore used the peptide chip to evaluate two inhibitors of c-Src, quercetin[27] and tyrphostin A47 (ref. 28), on a single substrate. Mixtures containing the kinase, [γ-^{32}P]ATP, and each inhibitor (at concentrations ranging from 1 nM to 100 μM) were arrayed onto monolayers under a layer of mineral oil (Fig. 3). After 4 hours at 37°C, the substrates were washed and quantified by phosphorimager analysis. Figure 4A shows concentration-dependent inhibition of enzyme

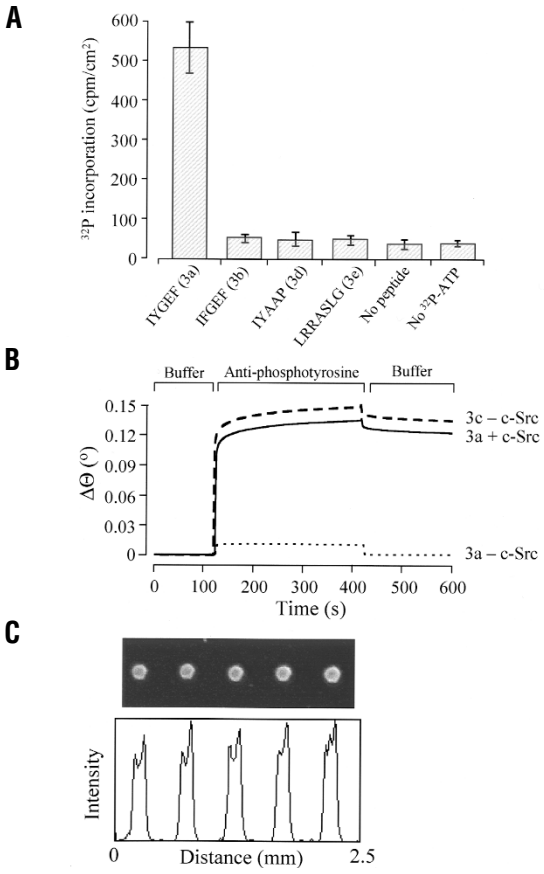

Figure 2. Characterization of the enzymatic phosphorylation of peptide chips by c-Src. (A) Substrates were treated with kinase and [γ-^{32}P]ATP for 2 h at 37°C, rinsed, and evaluated using scintillation counting. Incorporation of ^{32}P was observed on substrates presenting tyrosine conjugate **3a** but not on substrates presenting phenylalanine conjugate **3b** or no peptide. The amount of phosphorylation for each substrate is shown as the mean of results obtained using three chips on two separate occasions, and the error bars depict one standard deviation above and below the mean. (B) Peptide chips presenting tyrosine conjugate **3a** were treated with kinase or with an identical solution without kinase for 4 hours at 37°C. SPR, which reports the amount of protein binding by measuring a displacement angle in reflected light (ΔΘ), showed that an antibody against phosphotyrosine binds to monolayers treated with c-Src (solid line) but not to monolayers treated with a solution lacking the kinase (dotted line). Binding of the antibody to monolayers presenting phosphotyrosine conjugate **3c** (dashed line) resembled binding to monolayers after modification with the kinase. (C) Peptide chips presenting phosphotyrosine conjugate **3c** in an array format were incubated with anti-phosphotyrosine antibody and an AlexaFluor 532–labeled goat anti-mouse conjugate. Immunofluorescence micrographs and intensity profile of the monolayers show site-specific binding of the antibody to each element of the array.

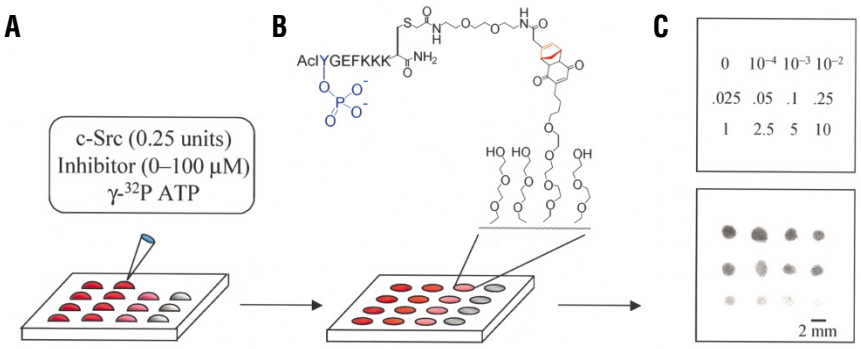

Figure 3. Scheme for the quantitative evaluation of kinase inhibitors in an array format. (A) A series of reaction mixtures containing kinase, [γ-^{32}P]ATP, and an inhibitor at a range of concentrations are arrayed onto a substrate presenting tyrosine conjugate **3a**. Mineral oil covers the substrate to prevent evaporation of the reaction mixtures. (B) After 4 h at 37°C, the substrate is rinsed and the incorporation of radioactive phosphate is evaluated using phosphorimaging. (C) Micrograph of an array prepared by spotting reaction mixtures containing kinase, [γ-^{32}P]ATP, and a series of concentrations of quercetin (0–10 μM). The concentration of inhibitor in each element of the array is shown above the micrograph.

activity by the two compounds. The inhibition data were fit to a 1:1 association model and analyzed to obtain K_i values of 0.31 μM and 0.17 μM for quercetin and tyrphostin A47, respectively.

For comparison, tyrphostin A47 and quercetin were also evaluated in a solution-phase format using cyclopentadiene conjugate 3a (0.1 mM) as the substrate. In these assays, quercetin produced half-maximal inhibition (IC_{50}) at a concentration of 15 μM, whereas tyrphostin had an IC_{50} of 8 μM. Both values agree with those reported previously[27,28]. It is notable that both inhibitors are 50-fold less effective in solution-phase assays. The difference in absolute inhibition values arises from the high concentration of peptide substrate (0.1 mM) used in solution-phase assays. Under these conditions, the peptide competes with the inhibitors for binding to the kinase, resulting in measurements that underestimate the potency of the inhibitor. In the immobilized format, however, the peptide is present in limiting amounts relative to the inhibitor. Data acquired using this format therefore reflect equilibrium binding of inhibitors to the enzyme in the absence of the peptide, permitting the direct determination of K_i values. We attempted to measure the K_i of each inhibitor in solution, but neither compound was purely competitive or noncompetitive over the concentration range used in this study.

To compare the solution and surface assays directly, we chose the PP1 inhibitor of c-Src, which is a purely noncompetitive inhibitor of Src-family kinases[24,29]. In solution-phase assays, this inhibitor gave an IC_{50} of 150 nM and a K_i of 15 nM. Inhibition assays using the monolayer substrate (Fig. 4B) gave a K_i of 39 nM, which is consistent with the value obtained in our solution-phase assays. The advantage of the chip-based assay is that it enables the reproducible, quantitative evaluation of inhibition in the absence of excess substrate. In addition, this assay format requires only minute quantities of both kinase and inhibitors, which often are available in small amounts and may have limited stability. The magnitude of signal in these experiments can be improved by using [γ-^{33}P]ATP, by allowing the reaction to proceed longer, or by analyzing the substrate using a ^{125}I-labeled secondary antibody. However, our results show that the signal-to-noise ratio of these experiments is sufficient for the characterization of inhibitors that have micromolar to nanomolar IC_{50} values in solution.

Application to peptide arrays. To demonstrate that our surface engineering approach is compatible with conventional microarray technologies, we used the Affymetrix 417 arrayer to immobilize into a microarray 10 peptides, biotin, and 2 carbohydrates (Fig. 5). Treatment of the arrays with an antibody against phosphotyrosine, an antibody against biotin, and concanavalin A allowed identification of the target ligands of each protein, indicating that the Diels–Alder-mediated immobilization was compatible with the arraying apparatus and that the immobilized ligands were capable of specific binding interactions.

The overall strategy we describe here has several practical characteristics that make it well suited to the preparation of peptide chips. First, the methods used to prepare the substrates are straightforward and reproducible. The required materials include gold-coated substrates, the hydroquinone and oligo(ethylene glycol)-terminated alkanethiols, and diene conjugates of the peptides. The Diels–Alder reaction provides an especially convenient and straightforward method for immobilization of peptides on the chip[20,22]. The peptides can be patterned into arrays using commercially available robotics. An additional benefit of these sub-

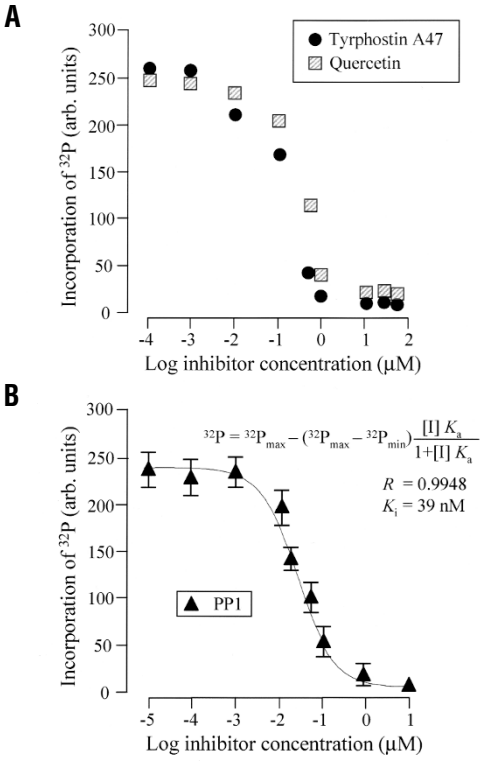

Figure 4. Evaluation of three inhibitors of c-Src on a single peptide chip. For each inhibitor, drops (0.25 μL) containing the kinase, [γ-^{32}P]ATP, and inhibitor (0–100 μM) were arrayed onto the chip as described in Figure 3. (A) Data for the inhibition of c-Src by quercetin (squares) and tyrphostin A47 (circles). The amount of phosphorylation decreased with higher concentrations of inhibitor and phosphorylation was completely blocked at inhibitor concentrations of 10 μM. The standard deviation for each data point is <10% of its value. (B) Inhibition experiment using the noncompetitive inhibitor PP1. The data were fit to a 1:1 association model (shown in inset) to give an inhibition constant of 39 nM. Similar analysis for tyrphostin A47 and quercetin gave inhibition constants of 0.17 μM and 0.31 μM, respectively. Data points represent the mean of results obtained from experiments carried out in duplicate on three separate occasions, and error bars depict one standard deviation above and below the mean.

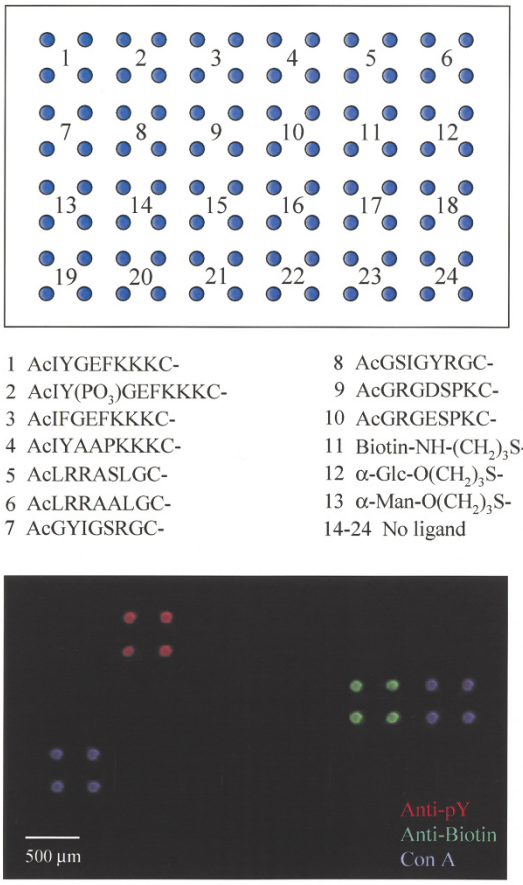

Figure 5. Microarray presenting multiple peptides and small molecules. Arrays were prepared by spotting 13 ligand–diene conjugates in quadruplicate onto benzoquinone-terminated monolayers. The array comprised 10 different peptides, biotin, 2 carbohydrates, and several regions that contained no ligand–diene conjugates (top and middle panels). Three identical arrays were incubated with anti-biotin antibody, anti-phosphotyrosine antibody, or the lectin concanavalin A and examined using the confocal array scanner. The fluorescence images were then false-colored and superimposed to generate a composite image (bottom panel). The anti-phosphotyrosine antibody binds only to phosphotyrosine residues in region 2, whereas the anti-biotin antibody binds only to biotin ligands in region 11 and concanavalin A binds only to carbohydrates in regions 12 and 13. In each case, fluorescence is observed only in regions of the chip presenting the appropriate analyte(s). This experiment demonstrates that the peptide chips are compatible with conventional microarray technology.

strates is their compatibility with the four principal techniques used to interrogate arrays. In this paper, we used assays based on radioactivity, fluorescence, and surface plasmon resonance spectroscopy. Other work has shown that monolayers on gold are excellent substrates for MALDI time-of-flight mass spectrometry[30]. Finally, the ability to rigorously control the density of peptides presented on the array by adjusting the density of quinone groups in the monolayer will be useful for optimizing levels of signal when preparing many identical arrays.

Comparison with other peptide chips. Our strategy for preparing peptide chips has two significant advantages over existing formats. The first derives from the ordered structure of the self-assembled monolayer, which provides a regular, homogeneous environment for immobilized peptide ligands[17,31]. The peptides therefore have equal activity toward soluble enzymes and are well suited to quantitative assays. The second advantage is the low to negligible levels of background signal. This property stems from the use of monolayers that present oligo(ethylene glycol) groups, which are highly effective at preventing the nonspecific adsorption of protein[15]. Many current formats for peptide and protein chip assays require that the substrate be blocked with an adsorbed layer of protein, which can prevent further nonspecific interactions with the substrate. The use of inert monolayers avoids the need for blocking procedures that can compromise the activities of immobilized peptides and lead to less reproducible assays.

Our system should be applicable to other assays that involve peptide arrays. In current work, we are preparing peptide arrays for the identification of protein–ligand complexes, for the evaluation of other enzymatic activities (including protease and phosphatase activities), and for the determination of optimal flanking sequences for enzyme substrates. Finally, our strategy—which relies on Diels–Alder-mediated immobilization to inert monolayers—should be well suited for preparing chips that present arrays of other small molecules[32,33], including carbohydrates and non-natural targets.

Experimental protocol

Reagents. ScintiVerse scintillation fluid and glass coverslips (no. 2, Corning) were obtained from Fisher Scientific (Pittsburgh, Pennsylvania). Tyrphostin A47 (G 213) and c-Src kinase were purchased from Calbiochem (La Jolla, CA). Antibodies were purchased from BD Biosciences (anti-phosphotyrosine) and Molecular Probes (AlexaFluor 532–labeled goat anti-mouse, AlexaFluor 594–labeled goat anti-mouse, and anti-biotin). [γ-^{32}P]ATP was obtained from ICN (Costa Mesa, CA). PP1 was purchased from New England BioLabs (Beverly, Massachusetts). Quercetin dihydrate, mineral oil (PCR grade), rhodamine-labeled concanavalin A, and all other reagents were purchased from Sigma. Peptides were synthesized on Fmoc-Rink MBHA resin as described previously[34]. Details of the synthesis of all diene conjugates will be reported in due course.

Preparation of substrates. Titanium (5 nm) and then gold (15 nm) were evaporated onto glass coverslips. SPR measurements used slides containing a 50-nm film of gold. Self-assembled monolayers were prepared by immersing the coverslips in a methanolic solution containing hydroquinone conjugate 1 (ref. 20) and penta(ethylene glycol) conjugate 2 (ref. 21) (10 µM in compound 1, 1 mM in total thiol). After 8 h, the substrates were rinsed extensively with absolute ethanol and dried under a stream of nitrogen. The monolayers were treated with a saturated aqueous solution of 1,4-benzoquinone for 5 min to oxidize the immobilized hydroquinone groups to the corresponding quinone. To immobilize the peptide conjugates to the monolayer, each substrate was inverted onto a small volume (20–100 µL, depending on the size of the substrate) of an aqueous solution of peptide conjugate 3a–e (2 mM in H_2O) on Parafilm. The substrates were kept in a humidified chamber at 37°C for 2 h, washed extensively with water, and dried under a stream of nitrogen before use.

Solution-phase kinase assays. Protein kinase assays were done according to the manufacturer's protocol for c-Src. Briefly, reaction mixtures containing ATP mix (10 µL of an aqueous solution containing 150 µM ATP, [γ-^{32}P]ATP (1.25 µL, 3.75 µCi/µL), and 30 mM $MgCl_2$), peptide (10 µL of a 0.1 mM solution in 50 mM HEPES, pH 7.5, containing 0.1 mM EDTA and 0.015% Brij 35) and kinase (0.5 units in 0.1 mg/mL BSA, 0.2% BME; 10 µL total volume) were incubated for 1 h at 37°C. Reactions were terminated by the addition of 10% H_3PO_4 (250 µL) and the mixture was applied to a phosphocellulose disc. The discs were washed extensively with 0.5% H_3PO_4, immersed in acetone, dried, and analyzed by scintillation counting. For inhibition experiments, inhibitor was included in the reaction mixture and the concentration of DMSO was adjusted to 2%.

Solid-phase kinase reactions. Kinase reaction mixtures were prepared as described above except that soluble peptide was omitted from the reaction mixture. This modified solution was added to substrates in a humidified chamber, and after a specified amount of time at 37°C, each substrate was washed with SDS (10% aqueous solution, 3 × 5 min) and water (3 × 5 min). Monolayers presenting radioactive phosphate were then exposed to a phosphor screen for 12 h or immersed in 5 mL of scintillation fluid. Analysis was done using a Molecular Dynamics (Sunnyvale, California) phosphorimager or a Beckmann (Fullerton, California) scintillation counter, respectively.

Nonisotopically labeled substrates were visualized by incubation for 1 h with an anti-phosphotyrosine antibody (10 μg/mL in PBS containing 0.1% BSA), followed by incubation for 30 min with an Alexa 532–goat anti-mouse IgG conjugate (10 μg/mL in PBS containing 0.1% BSA). After each incubation, substrates were washed with PBS (3 × 5 min). Fluorescence was analyzed using a Typhoon Imaging system and ImageQuant software (Molecular Dynamics) or with an AffyMetrix 428 ArrayScanner.

For array experiments, substrates were covered with a layer of mineral oil and reaction mixtures (0.25 μL) containing [γ-^{32}P]ATP (1.25 μL, 3.75 μCi/μL) were pipetted onto monolayers using the Biomek (Beckmann Coulter, Fullerton, California) 2000 robot. Reactions were incubated at 37°C for 4 h, and the mineral oil layer was removed by washing with hexanes. The surface was then rinsed with 10% SDS (3 × 5 min) and water (3 × 5 min). Phosphorylation was detected by exposure to a phosphor screen for 12 h and analyzed using ImageQuant.

For inhibition experiments, reaction mixtures containing a series of concentrations of inhibitors were prepared in 96-well microtiter plates. Each reaction mixture contained 2% DMSO (final concentration). Reaction mixtures (0.25 μL) containing [γ-^{32}P]ATP (1.25 μL, 3.75 μCi/μL) were spotted onto monolayers overlaid with mineral oil, and after incubation for 4 h at 37°C, the substrate was washed and analyzed as described above.

Preparation of multicomponent arrays. Solutions of diene conjugates (2 mM in 1:1 H_2O/glycerol) were arrayed onto three identical benzoquinone-terminated monolayers using the GMS 417 arrayer. The substrates were kept in a humidified chamber for 2 h, rinsed with distilled water and ethanol, and dried under a stream of nitrogen. Each slide was then inverted onto a solution of anti-phosphotyrosine antibody (10 μg/mL in PBS), anti-biotin antibody (1 μg/mL in PBS), or rhodamine-labeled concanavalin A (100 μg/mL in PBS containing 0.1 mM $CaCl_2$). After 30 min, the slides were washed with PBS (3 × 5 min). Slides stained with anti-biotin and anti-phosphotyrosine were incubated with Alexa 532–conjugated and Alexa 594–conjugated secondary antibodies, respectively, for 30 min and washed again with PBS (3 × 5 min). All monolayers were imaged using the confocal array scanner.

Surface plasmon resonance spectroscopy. SPR measurements were done using a BIACore (Piscataway, New Jersey) 1000 instrument. Monolayer substrates were incorporated into the BIACore cassettes by removing the manufacturer's substrate and gluing the chip into the cassettes using a two-part epoxy (Devcon)[35]. Measurements were obtained using PBS saline (pH 7.4) as the running buffer and are reported as changes in resonance angle (Δθ), where 1° = 10,000 RU. The bulk refractive index of the antibody solution was measured by injection of the solution over a monolayer presenting penta(ethylene glycol) groups alone.

Acknowledgments

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