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Steady-State of an Enzymatic Reaction is Dependent on the Density of Reactant

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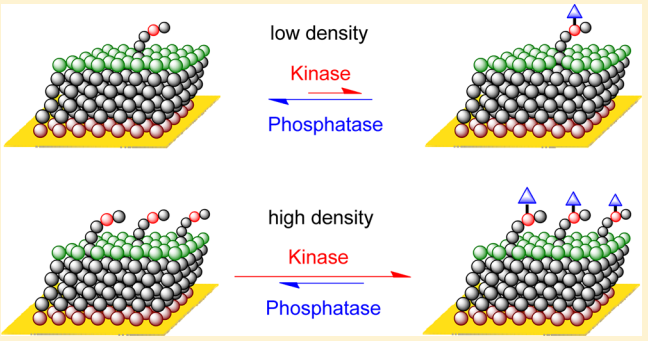
Supporting Information

ABSTRACT: The post-translational modification of proteins is controlled by the relative activities of two opposing enzymes. For example, the extent of phosphorylation of tyrosine residues reflects the balance of a kinase and a phosphatase enzyme. The present article uses as a model system a self-assembled monolayer that presents a peptide that can be phosphorylated by Abl kinase and subsequently dephosphorylated by Lambda phosphatase. Treatment of monolayers with a reaction mixture containing both enzymes reveals that the steady-state level of peptide phosphorylation is dependent on the density of the peptide. Using identical reaction mixtures, surfaces that presented the substrate at high density led to a phosphorylated peptide at steady-state, whereas surfaces that presented the substrate at low density led to unphosphorylated peptide at steady-state. This dependence owes to an autocatalytic phosphorylation reaction that operates at high densities of substrate. This work provides an example of an interfacial reaction that has properties that have no analogue in the corresponding solution phase reaction. It also provides a model system that is relevant to understanding mechanisms that regulate signaling at the cellular membrane.



Reactions that occur at the solid−liquid interface can display features that are distinct from those performed in solution. Recent examples include: the rapid polymerization of long-chain esters of amino acids at the air−water interface;[1] the catalytic hydrogenation of carbonyls by a rhodium complex tethered to a Langmuir−Blodgett film;[2] the *syn* selectivity of hydrohalogenation reactions performed at alumina surfaces;[3] and a switch in protein binding specificity for a carbohydrate that was immobilized either at low or high density.[4] Studies of surface-dependent reactivities, particularly for those cases that have no analogue in the corresponding solution-phase reaction, are interesting and of potential importance. In this article, we report an enzyme-catalyzed phosphorylation reaction that changes its steady-state preference for the substrate and product when the density of the immobilized reactant is changed. We treat a monolayer that presents a peptide substrate with a mixture of kinase and phosphatase and we show that for low densities of peptide, the unphosphorylated peptide dominates at steady-state whereas for high densities of peptide, the phosphorylated form dominates.

Our system is based on the Abelson tyrosine kinase (Abl), which has both a catalytic domain and a Src homology 2 (SH2) domain that binds to the phosphopeptide product of the phosphorylation reaction (Figure 1). In earlier work, we found that when a peptide substrate for Abl was immobilized to a self-assembled monolayer (SAM) of alkanethiolates on gold, the enzymatic phosphorylation proceeded autocatalytically—that is, the rate increased as the reaction progressed. The rate acceleration of approximately 30-fold was due to binding of the SH2 domain to the phosphopeptide product, which served to recruit the kinase to nearby substrates.[5] We further showed that the reaction product propagated in a spatially organized manner, since the reaction is most rapid at the boundary between the peptide substrate and the phosphopeptide product.[5,6] Finally, the autocatalytic reaction requires that the peptide is present at densities greater than 1% (relative to total alkanethiolate in the monolayer), because at lower densities the bound kinase is not within reach of other peptide substrates.

We reasoned that this density dependence could be used to control the direction of the phosphorylation reaction when both a kinase and a phosphatase were present in reaction solution (Figure 2). For example, for monolayers presenting a high density of peptide substrate (in this work, where 5% of the alkanethiolates present peptide), concentrations of enzymes can be selected such that the kinase would phosphorylate the peptide in the autocatalytic mode, and with a rate that was greater than that of the opposing phosphatase leading to complete phosphorylation of the peptide at steady-state. For lower densities of substrate (0.2%), however, where the kinase does not exhibit the autocatalytic reaction, the rate for phosphorylation would be slower than that for dephosphorylation and the peptides would largely exist in nonphosphorylated form at steady-state. In this way, the treatment of an immobilized

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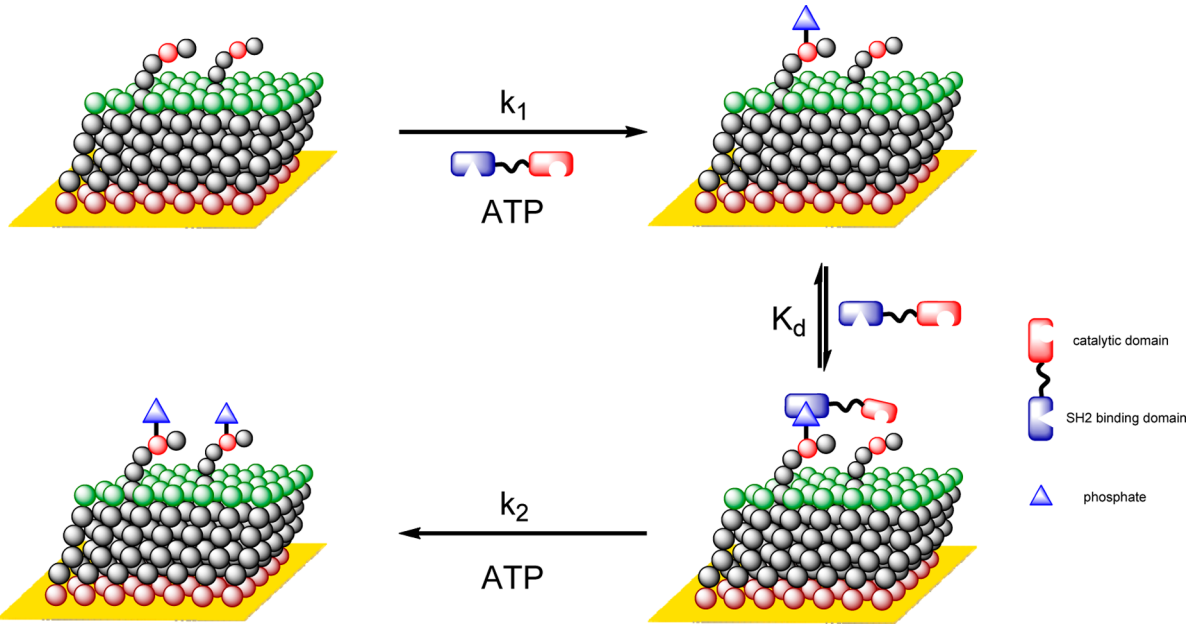

Figure 1. This work uses self-assembled monolayers presenting a peptide substrate for Abl kinase and a phosphatase enzyme. The kinase phosphorylates the peptide in an autocatalytic process that starts with initial phosphorylation of a peptide with a rate constant k_1. This phosphopeptide then binds to the SH2 domain of Abl (in blue), with a dissociation rate constant K_d, where it positions the catalytic domain to more rapidly phosphorylate neighboring substrates with an effective rate constant k_2.

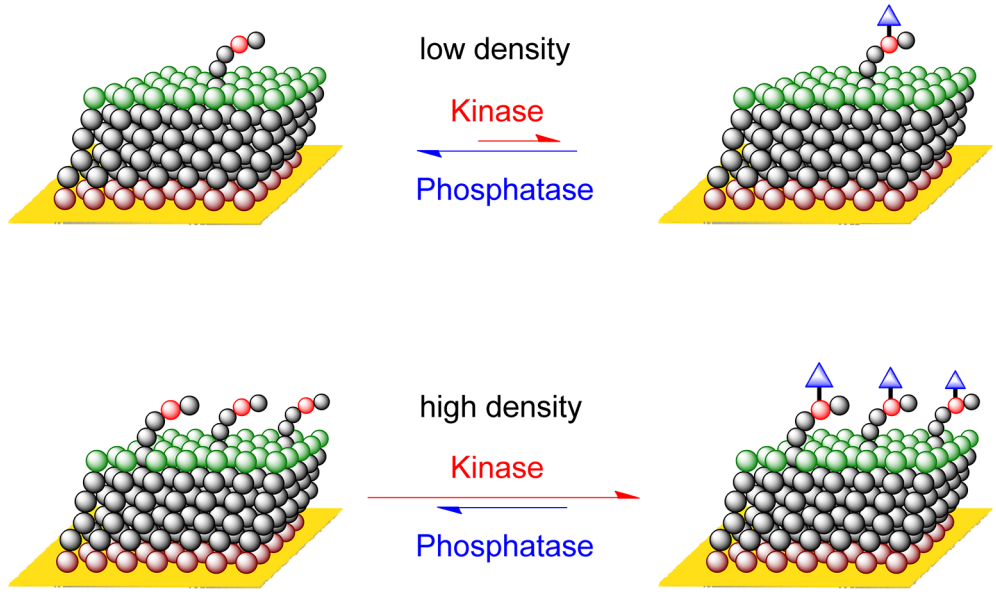

Figure 2. Extent of phosphorylation of the immobilized peptide at steady-state depends on the density at which the substrate is immobilized. At low densities (top), the phosphatase activity exceeds that of the kinase, and the peptide primarily exists in the nonphosphorylated form. At high densities (bottom), the autocatalytic process leads to a faster phosphorylation reaction, and a steady-state that prefers the phosphorylated form of the peptide. The red circles represent the tyrosine residue of the peptide and the blue triangles represent the phosphate group.

peptide with opposing enzymes will, under identical conditions, give different products for surfaces that present the peptide at high or low density. Further, a surface that can be dynamically altered to change the density of immobilized peptide would be expected to respond with a change in the phosphorylation state of the peptide.

The structure of the monolayer used in the first part of this work is shown in part A of Figure 3 and was prepared by immobilizing the cysteine-terminated peptide AIYENPFARKC to a monolayer presenting maleimide groups — at densities of 5% or 0.2% — against a background of tri(ethylene glycol) groups.[7] We used this peptide because it is a suboptimal catalytic substrate for Abl kinase, but once phosphorylated to give AIpYENPFARKC (which we abbreviate as Y and pY, respectively), it has high affinity for the Abl SH2 domain.[8,9] The glycol groups render the monolayer inert to protein adsorption. To perform reactions, we applied solutions containing Abl kinase, ATP, and Lamda protein phosphatase (λPP) to the monolayers, allowed the reactions to proceed at 30 °C, and then rinsed and analyzed the surfaces using matrix-assisted laser desorption-ionization mass spectrometry (i.e., the SAMDI method) to determine the extent of phosphorylation (part A of Figure 3).[10−12] We determined the yield

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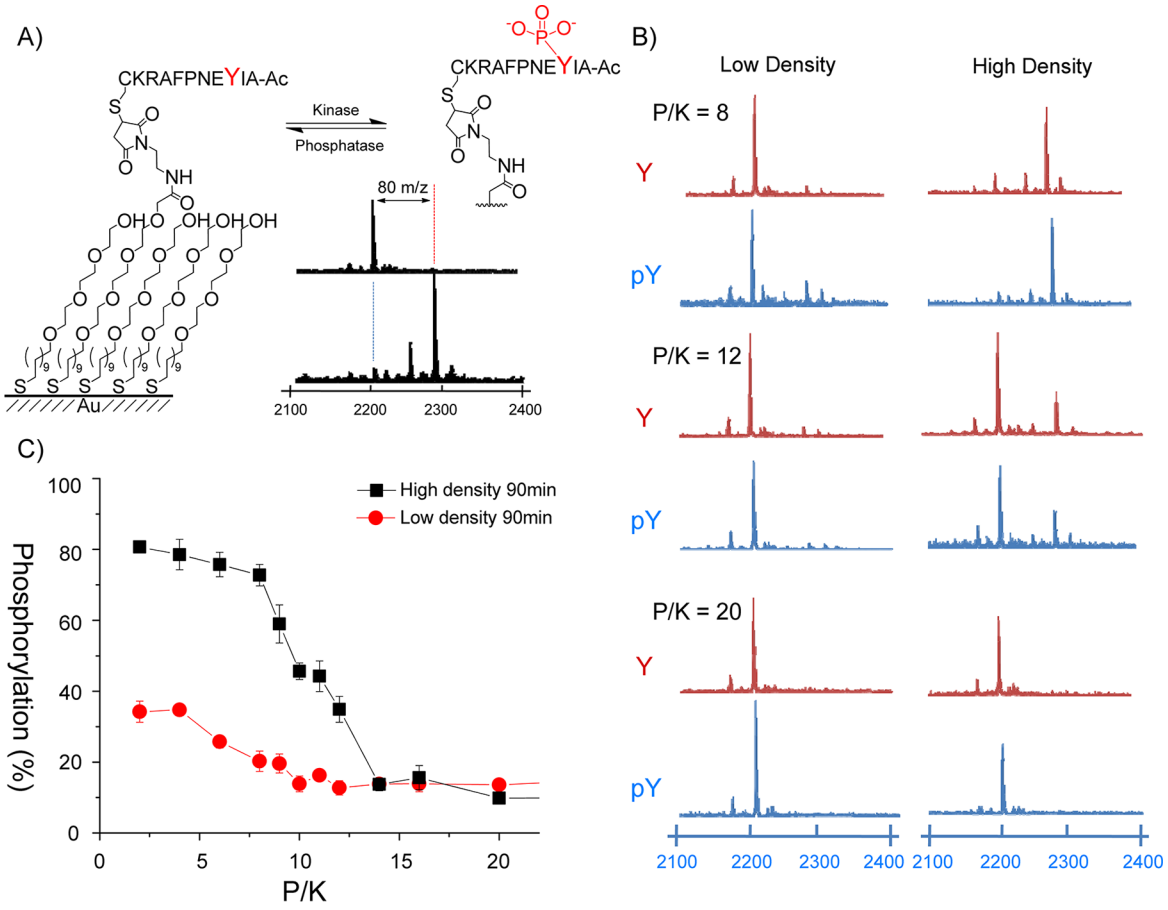

Figure 3. (A) Treatment of a monolayer that presents a peptide substrate with Abl kinase results in phosphorylation of the peptide. SAMDI mass spectra of the monolayer before and after the kinase treatment reveal the 80 Da mass shift expected for phosphorylation. Likewise, treatment of the phosphorylated peptide with phosphatase is accompanied by a loss in mass of 80 Da. (B) Monolayers were prepared having either peptide (Y, red) or phosphopeptide (pY, blue) immobilized at low (left) or high (right) density and treated with defined ratios of phosphatase and kinase (P/K). The surfaces were rinsed and analyzed by SAMDI mass spectrometry. (C) The fraction of peptide that is phosphorylated at steady-state is shown for a range of P/K ratios for monolayers presenting peptide at high (black squares) and low (red circle) densities.

by dividing the intensity of the peak for the phosphopeptide by the sum of the intensities of the peaks for the phosphopeptide and the peptide substrate. We also performed all reactions on two monolayers—one that initially presented the peptide substrate (Y) and one that presented the phosphopeptide substrate (pY)—and found that both surfaces gave the same products, confirming that the reactions achieved steady-state (part B of Figure 3).

We treated four series of monolayers — which differed in that they presented the peptide in unmodified or in phosphorylated form and at high or low density — with solutions containing both the kinase and the phosphatase. We used eleven ratios of phosphatase to kinase (the P/K value) as described in the Supporting Information. For the monolayers that presented peptide at low density (0.2%), we found that all reactions gave only unphosphorylated products (part C of Figure 3). That is, on these surfaces, the phosphatase activity overwhelmed the kinase activity, as we expected when the kinase could not use the autocatalytic mode. For monolayers presenting peptide at the high density (5%), the steady-state product depended on the ratio of the opposing enzymes. When P/K was 20, we observed complete dephosphorylation of the peptide at steady-state, whereas a P/K ratio of 2 gave phosphorylated peptide. Intermediate ratios of the enzymes gave intermediate proportions of the two forms of the peptide. As noted earlier, these findings did not depend on whether the immobilized peptides were initially phosphorylated demonstrating that the observed products are representative of the steady-state.

We next demonstrate control over the reaction with a monolayer that initially presents substrate at low density but that can be switched to a state that presents substrate at high density. We prepared a monolayer by coimmobilizing a mixture of the peptide and 3-butyne-1-thiol to a monolayer presenting the maleimide group at a density of 5%. We adjusted the relative amounts of the two molecules to give monolayers having the peptide present at an initial density of approximately 0.2% and therefore the alkyne present at a density of approximately 4.8%. The latter group can subsequently be treated with an azido-terminated peptide to increase the density of immobilized peptide to 5% (Figure 4A). We treated the initial monolayer (with peptide at 0.2% density) with a reaction cocktail having P/K of 1 and found that the extent of phosphorylation of the peptide at steady-state was approximately 20%. We then treated the monolayer with $[Cu(CH_3CN)_4]PF_6$ and the azido-terminated peptide to immobilize peptide at higher density, and again treated the monolayer with the reaction cocktail. We then found that the approximately 80% of the peptide was phosphorylated at steady-state (part B of Figure 4). Hence, the steady-state phosphorylation of the peptide can be changed by increasing the density of peptide on the monolayer. We repeated this experiment for several reaction cocktails having a range of

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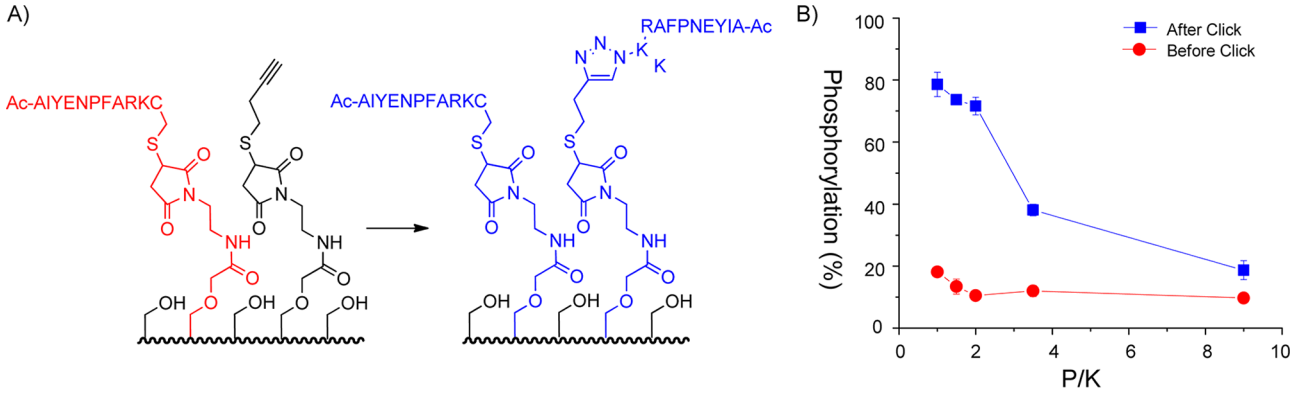

Figure 4. (A) Design of monolayers that can be switched to present higher densities of peptide. A monolayer presenting peptide and terminal alkynes is treated with $[Cu(CH_3CN)_4]PF_6$ and an azido-terminated peptide to increase the density of the peptide. (B) The fraction of peptide that is phosphorylated at steady-state is shown for a range of P/K ratios for monolayers presenting peptide before (red circles) and after immobilization of additional peptide (blue squares).

P/K values and we found that as the phosphatase activity increased, the ability to switch the steady-state intermediate suffered.

In this work, we demonstrate an interfacial reaction that has the interesting property that the steady-state intermediate established by the action of two opposing enzymes is itself dependent on the density of the enzyme substrate. This property stems from an autocatalytic phosphorylation that increases the rate of phosphorylation by approximately thirty-fold when the peptide is present at high density. This autocatalytic reaction requires that the density of peptide be greater than 1% (relative to total alkanethiolates).[5] Hence, by switching our densities from 0.2% to 5%, we can cross the threshold required for initiating the autocatalytic process. The phosphatase enzyme, by contrast, has a rate constant for its reaction that is not dependent on the density of peptide. This is the basis for altering the relative kinase and phosphatase activities as the density is varied. It is significant that this effect requires that the peptides be immobilized to prevent diffusion and therefore it has no counterpart in the corresponding solution-phase reaction.

It is interesting that, even for the highest ratio of P/K activity, we observe about 10% of the peptides present in the phosphorylated form. We believe that this fraction could be due to a statistical clustering of peptides giving regions having a higher local density, or phase-separated domains that are enriched in the peptide-terminated alkanethiolates. The use of the tri(ethylene glycol)-terminated monolayers makes direct observation of these domains difficult to observe experimentally. Even so, the dependence of the steady-state form of the peptide on the average density of the peptide and the ratio of P/K activity is consistent with a mechanistic model wherein peptides present at a threshold density can be phosphorylated in an autocatalytic process.

We note that this behavior has a strong analogy to biological processes that occur at the cell membrane, where the phosphorylation of proteins is also determined by the action of opposing activities that generate a steady-state level of product. The signaling of tyrosine receptor kinases often goes by way of dimerization of receptors,[13] conformational changes in the receptor,[14] or clustering into multireceptor complexes.[15] However, our system appears unique in that it requires clustering of the receptor (beyond dimerization) but does not require additional receptors to be present in the complex. We are unaware of natural systems with this feature but recently demonstrated that a synthetic receptor is able to undergo phosphorylation when clustered by an extracellular bead.[16] We believe that this and related model systems will be important for elucidating the physical organic chemistry of complex biological reactions.

EXPERIMENTAL SECTION

Synthesis of Peptides. Peptides were synthesized at 0.1 mmol scale each on Fmoc-Rink amide 4-methylbenzhydrylamine resin (AnaSpec. Inc., San Jose, CA). The Fmoc group was removed by treating with 20% piperidine in DMF (15 min + 5 min). Incorporation of amino acids (4 equiv) was achieved with PyBop (4 equiv.) and *N*-Methylmorpholine (4 equiv) in DMF for 30 min. Protected amino acids (4 equiv) was coupled with HOBt (4 equiv) and DIC (4 equiv) in DMF for 2 h. For preparation of the azido-terminated peptide substrate, the side chain amino group of lysine was protected with the Alloc group. After completion of the solid synthesis on resin, the Alloc protecting group was selectively removed using $Pd(PPh_3)_4$ (0.1 equiv) in the presence of $PhSiH_3$ (20 equiv) in CH_2Cl_2 under Argon (2 × 20 min, 25 °C) and then coupled with 5-azido pentanoic acid before cleavage from the resin using the standard protocol. All peptides were purified by reverse phase HPLC on a C18 column (water, acetonitrile).

Preparation of SAMs Presenting Maleimide Groups. The maleimide-presenting SAMs were prepared as previously reported.[7] Gold-coated coverslips (4 nm Ti, 22 nm Au for most experiments, 4 nm Ti, 50 nm Au for click reactions) were immersed in an ethanolic solution containing a symmetric disulfide presenting tri(ethylene glycol) groups and an asymmetric disulfide presenting one maleimide group and one tri(ethylene glycol) group at different ratios (in case of 5% maleimide density, a molar ratio of 9:1 was used) overnight with a total concentration of disulfide of 0.2 mM.

Kinase/Phosphatase Assay. Phosphate buffered saline (pH 7.4) containing cysteine-terminated peptides (0.2 mM) were applied to SAMs and incubated at room temperature for 30 min to immobilize peptides. The monolayers were rinsed with distilled water and ethanol and dried under nitrogen. For the assay, Abl kinase (New England Biolab, Ipswich, MA) was diluted to 1−2 units/μL in buffer (50 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, pH 7.5) with 1 mM ATP (supplemented with 1 mM Mn^{2+} if Lamda Protein Phosphatase (New England Biolab, Ipswich, MA) was added) and 1−2 μL was applied to each monolayer and incubated at 30 °C for 90 min.

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Mass Spectrometry. Monolayers were treated with matrix (2,4,6-trihydroxyacetophenone, 30 mg/mL in acetonitrile), dried, and analyzed by SAMDI-MS to produce a mass spectrum for each gold spot. Mass analysis was performed using a 4800 MALDI-TOF/TOF (Applied Biosystems, Framingham, MA). A 355 nm Nd:YAG laser was used as a desorption/ionization source, and all spectra were acquired with 20 kV accelerating voltage using positive reflector mode. The extraction delay was 450 ns, 3000 laser shots were applied, and the entire surface of the gold spot was sampled.

Semi-Quantitative Analysis and Ionization Efficiency of Peptides. For quantification, the extent of phosphorylation was determined based on the relative peak intensity of product and substrate on SAMDI spectra: Yield = $I_p/(I_p + I_s)$, where *p* refers to the phosphorylated peak and *s* refers to the parent peak. To calibrate the ionization efficiency of parent peptides and phosphorylated peptides, maleimide-conjugated phosphorylated and parent peptides were first prepared and then mixed at a series of ratios to form monolayers with defined densities of maleimide-conjugated phosphorylated and parent peptides. SAMDI mass spectra of these monolayers showed that the determined yields were within 10% of the actual yields.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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