Acc. Chem. Res. **2003,** *36,* 401−409

Chemical Probes of Signal-Transducing Proteins

DAVID S. LAWRENCE
Department of Biochemistry, The Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, New York 10461

Received October 8, 2002

Ⓦ This paper contains enhanced objects available on the Internet at http://pubs.acs.org/accounts.

ABSTRACT

Protein kinases are key participants in signal transduction pathways. A direct assessment of the relationship between the activity of any given protein kinase and the corresponding cellular phenotype has proven challenging. This is due to the large number of protein kinases encoded by the human genome coupled with intracellular temporal and spatial constraints that appear to further regulate the ultimate response of a cell to a stimulus. Our work has focused on the development of chemical probes to address the complexities associated with protein kinase-mediated cell signaling. These include the acquisition of highly selective substrates and inhibitors for specific members of the protein kinase family, the design and synthesis of light-activated signaling proteins and their corresponding inhibitors, and the preparation of fluorescent reporters of intracellular protein kinase action.

Introduction

Protein kinases catalyze the transfer of the γ-phosphoryl group of ATP to the hydroxyl moieties of serine, threonine, and tyrosine in intact proteins. This deceptively modest reaction serves as a cornerstone for the extraordinarily complex phenomenon known as signal transduction, the biochemical process by which information is transmitted between different cellular sites. For example, the binding event between growth factor and receptor on the cell surface is signaled to the nucleus via protein kinase-mediated pathways. In response to this signal, genes are transcribed, and the cell prepares itself for division. Mitosis is subsequently driven by a fine choreography of temporally and spatially regulated signaling pathways that ensure the myriad of biochemical processes required for replication occur in their proper chronological order. In short, signal transduction serves as a biochemical mechanism that drives an extraordinary array of biological phenomena. However, it would be simplistic to view signaling pathways as the molecular equivalent of the interstate highway system. The latter is fixed both in time and space. By contrast, kinase-mediated pathways not only evolved to rapidly form in response to some environmental stimulus, but their role in cellular homeostasis is dependent upon their rapid disassembly once the environmental signal has been acknowledged. Furthermore, the nature of the cellular response is dependent upon when and/or where a specific pathway is activated as well as upon what other pathways may be simultaneously operating. Much of our work has focused on the synthesis and evaluation of molecular probes designed to assess the complex intracellular action of signaling proteins. In this Account I will highlight four areas that have attracted our attention, namely, the design and acquisition of (a) kinase-specific substrates and inhibitors, (b) ligands that block signal complex assembly, (c) light-activated signaling proteins and inhibitors, and (d) fluorescent reporters of protein kinase activity.

Protein Kinase-Specific Substrates and Inhibitors

The substrate specificity of any given protein kinase is typically defined as the preferred amino acid sequence that envelops the serine, threonine, or tyrosine residue phosphorylated by the enzyme (consensus recognition sequences).[1] In addition, protein kinases are typically divided into two families on the basis of their active site specificity: those that phosphorylate the aromatic phenol of tyrosine and those that catalyze the phosphorylation of the aliphatic alcohols of serine and threonine. We have found that active site substrate specificity is not limited to the three naturally occurring alcohol-containing amino acids specified by the genetic code, but encompasses an extraordinary array of unnatural hydroxylated residues.

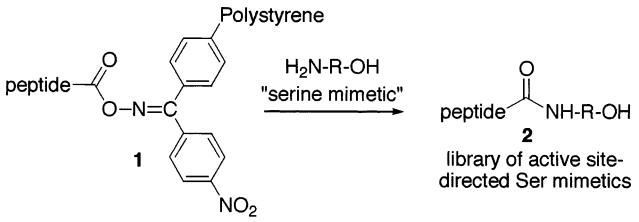
Many protein kinases will phosphorylate peptides containing C- and N-terminal-appended serine and threonine residues.[2,3] This simple observation provides a rapid entry into the preparation of active site-directed peptides containing a wide assortment of serine/threonine mimetics. We have prepared libraries of alcohol-appended peptides using either tBoc[2]- or Fmoc[3]-based solid-phase peptide synthesis methodologies. For example, the tBoc protocol employs Kaiser's oxime resin **1**. The active site-directed peptide is linked to the synthesis bead via a relatively labile oximate ester (Scheme 1). Consequently, the peptide can be simultaneously condensed with the serine mimetic and displaced from the resin support (Scheme 1). An attribute apparent from the synthetic strategy portrayed in Scheme 1 is that the α-carboxylate of the serine analogue is not required since the analogue is not inserted into the interior of the peptide. Since the chiral center and the α-carboxylate are missing, the synthesis of these serine analogues is exceedingly straightforward.

The cAMP-dependent protein kinase (PKA) catalyzes the phosphorylation of the C-terminal Ser in acetyl-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ser-amide.[2] Consequently, the Ser moiety is easily replaced with a library of nonnatural aminoalcohols using the synthetic strategy outlined in Scheme 1. PKA phosphorylates a structurally diverse assortment of alcohol-bearing residues, including achiral

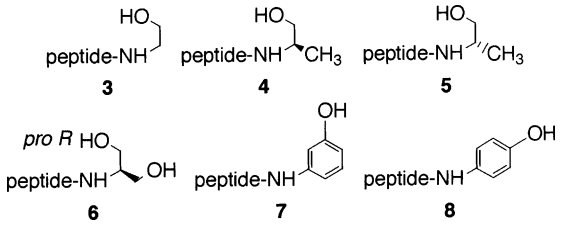
David S. Lawrence received his B.S. in Biological Sciences at the University of California at Irvine. However, his undergraduate research experience was in the general area of organic synthesis under the direction of Harold W. Moore. He earned his Ph.D. from UCLA in natural products synthesis with Robert V. Stevens and was then a postdoctoral fellow in the laboratory of E. Thomas Kaiser at the Rockefeller University. In 1985 he joined the Department of Chemistry at SUNY Buffalo as an Assistant Professor of Chemistry, subsequently rose through the academic ranks, and, in 1995, moved to the Albert Einstein College of Medicine, where he is currently a Professor of Biochemistry.

10.1021/ar020132s CCC: $25.00
Published on Web 02/19/2003

Scheme 1



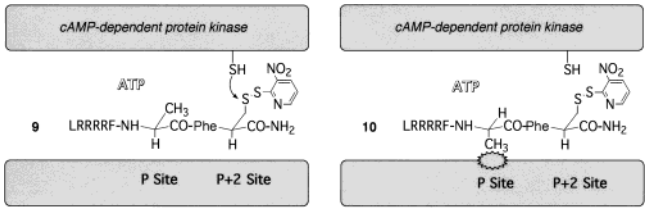
derivatives such as **3**, and α-substituted derivatives, such as **4**. However, PKA is unable to phosphorylate residues



containing an α-configuration analogous to that present in D-amino acids (e.g., **5**). This discriminatory behavior suggested that PKA should distinguish between prochiral hydroxyl moieties that are positioned on the same residue (**6**). Indeed, PKA only phosphorylates the *pro*-R hydroxymethylene in **6**.[4]

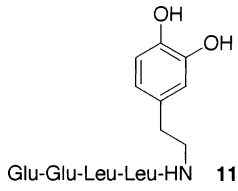
We've also assessed the active site specificity of the cGMP-dependent protein kinase (PKG)[5] and various protein kinase C (PKC) isoforms[3]. PKA, PKC, and PKG share a strong sequence homology, and all three comprise what is commonly referred to as the "ACG" subfamily of protein kinases. Not surprising, these enzymes display overlapping sequence specificities with respect to both substrate and inhibitor peptides. However, their active site specificities are remarkably different. For example, whereas PKA is unable to phosphorylate alcohol-bearing residues that possess an α-stereocenter corresponding to that present in D-amino acids (e.g., **5**), both PKC[3,6] and PKG[5] readily phosphorylate residues containing this configuration. Furthermore, the differences in active site specificity between these otherwise closely related protein kinases are not just limited to stereochemical biases. For example, PKC phosphorylates meta- (**7**) and para-substituted (**8**) phenols, whereas PKA and PKG do not.[7]

Given the intense interest in developing inhibitory agents that discriminate between PKA and PKG, we wondered whether the unique active site specificities of these two enzymes could be used to devise a PKG-selective inhibitor or inactivator. An example of the latter is illustrated with affinity labels **9** and **10**.[8] The amino acid



sequence Leu-Arg-Arg-Arg-Arg-Phe-Ser- is recognized by both PKA and PKG. The affinity label **9** was devised by replacing the phosphorylatable Ser ("P position") with an L-Ala and appending an electrophilic disulfide moiety at the P + 2 position. We anticipated that the reactive disulfide in **9**, when enzyme-bound, would be aligned adjacent to an active site cysteine residue present in both PKA and PKG. Indeed, both PKA (K_I = 25.4 M) and PKG (K_I = 15.1 M) suffer time-dependent inactivation upon exposure to **9**. By contrast, the corresponding affinity label **10** is designed to selectively inactivate PKG but not PKA. Compound **10** contains a D-alanine-moiety at the former serine position, and residues of this configuration are not accommodated within the active site of PKA. Therefore, the reactive disulfide at P + 2 cannot be properly positioned to covalently modify the active site cysteine. Indeed, even at concentrations as high as 1 mM, compound **10** fails to inactivate PKA. However, since PKG does accommodate D-residues at the P position, affinity label **10** serves as an excellent time-dependent inactivator (K_I = 21.1 M) for this protein kinase. We employed an analogous strategy to devise a simple D-alanine-containing nonapeptide that serves as a selective reversible PKG inhibitor.[9,10] This peptide has been used to demonstrate that PKG plays a key role in the signaling pathways responsible for memory and learning.[9,11]

Tyrosine-specific protein kinases[12,13] likewise phosphorylate a remarkable array of unnatural alcohol-containing residues. However, perhaps the most interesting aspect of the active site substrate specificity of the tyrosine kinase Src is what the enzyme will not phosphorylate.[14,15] The C-terminal-appended L-dopamine derivative **11** is resistant to phosphorylation; yet the presence of the catechol moiety enhances enzyme affinity by more than 30-fold.[14]

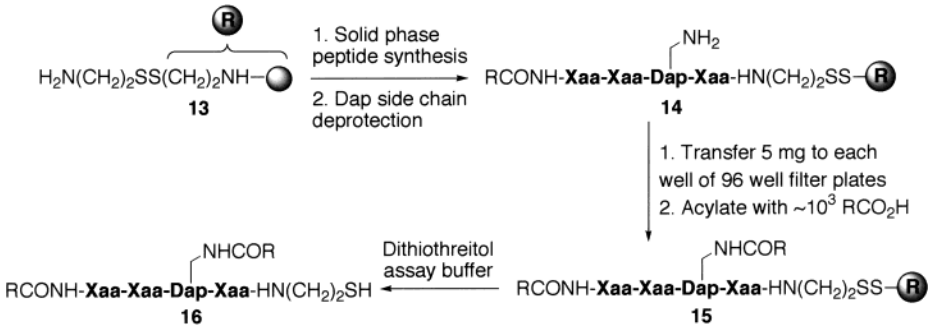


The corresponding L-Dopa-containing peptide, Glu-Glu-Leu-Leu-Dopa-Gly-Glu-Ile (**12**) is an even more potent nonphosphorylatable reversible inhibitor of Src. Interestingly, the catechol element present in **11** and **12** is also found in several tyrosine kinase inhibitors derived from natural products[16] and was subsequently identified as a lead structural motif in a small molecule library screened against Src.[17]

From Consensus Sequence Peptides to High-Affinity Ligands

In general, signaling proteins tranduce information by recognizing and binding to specific amino acid sequences on other proteins (i.e., via the assembly of signaling complexes). However, since protein signaling complexes are transient in nature, the protein−protein interactions that mediate the assembly of these complexes are not characterized by high stability constants. Consequently, the consensus sequences that drive signaling complex

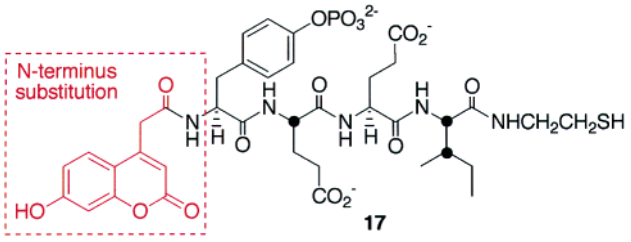
Scheme 2



formation tend to display a modest affinity (generally K_D > 1 μM) for their intended protein target. Is it possible to develop a general synthetic strategy to convert weakly binding consensus sequences into high affinity ligands? The parallel synthesis approach outlined in Scheme 2 was designed to address this question.

The overall strategy employs peptides that contain a (L)-2,3-diaminopropanoic acid moiety (Dap) at specific sites along the consensus sequence peptide chain.[18] Only those residues that contribute little to the binding event are replaced with the Dap moiety. Once the Dap-containing peptides have been prepared, the side chain Dap amine is deprotected (**14**) and the resin-appended peptide transferred in small quantities to individual wells of multiwell filter plates. The free amine-containing Dap functionality on the peptide-resin in each well is subsequently condensed with one of ~10^3 different carboxylic acids to furnish **15**. Upon removal of any remaining protecting groups, the peptides are released from the resin with assay buffer (dithiothreitol) and vacuum filtered into a multiwell plate in an assay ready form (**16**). This Scheme 2 protocol can be easily automated and iteratively applied to different sites along the peptide chain.

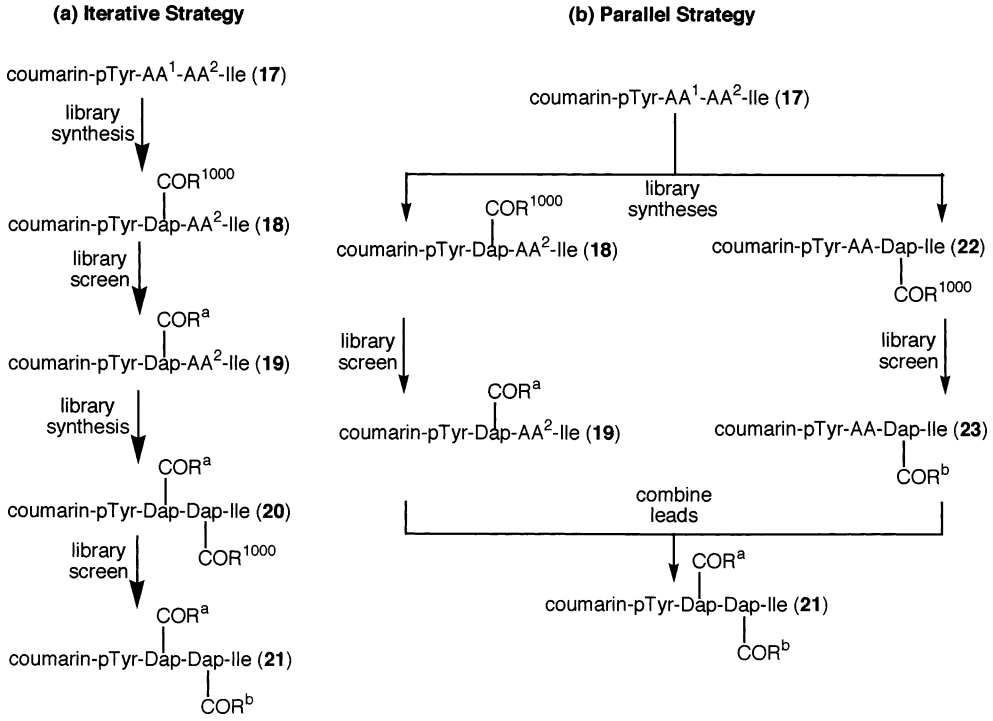
The Lck SH2 domain binds the consensus peptide acetyl-pTyr-Glu-Glu-Ile-amide with a K_D of 1.3 μM.[19] Since conventional amino acid residues on the N-terminal side of the pTyr moiety contribute little binding energy to the SH2/consensus peptide complex,[20] we employed the strategy outlined in Scheme 2 to identify a non-amino-acid replacement at the N-terminus that can productively engage the SH2 surface. The lead coumarin-containing compound (**17**) was identified from the ~10^3 member library via an enzyme-linked immunosorbent assay (ELISA).[19] An unexpected bonus associated with the coumarin moiety is that its fluorescence is not altered when bound to the Lck SH2 domain. Consequently, the K_D of the peptide **17**/SH2 domain complex can be directly determined via equilibrium dialysis. The affinity of **17** for



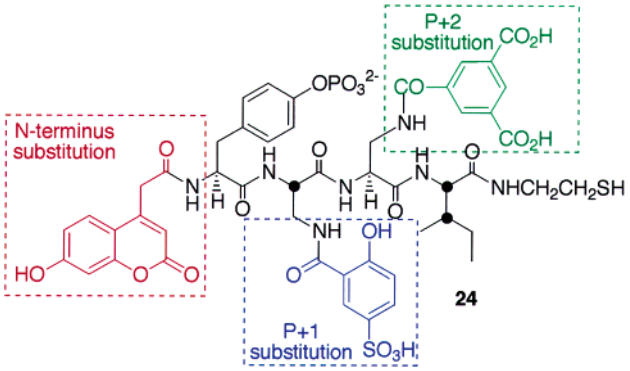
the Lck SH2 domain is nearly 2 orders of magnitude greater than that of the unmodified consensus peptide acetyl-pTyr-Glu-Glu-Ile-amide (i.e., 35 nM vs 1.3 μM).[19]

With an affinity promoting substituent at the N-terminus in hand, we turned our attention to identifying replacements for the two Glu residues at the P + 1 and P + 2 positions, residues that only weakly interact with the Lck SH2 surface.[20] Two possible synthetic strategies are outlined in Scheme 3. The "iterative" strategy depicted in Scheme 3a utilizes a stepwise approach which first identifies a modified Dap residue at one of the two positions (e.g., **18** to **19**) and then subsequently, with the P + 1 modified Dap moiety in place, screens for the optimal substituent on the Dap residue at the adjacent position (i.e., **20** to **21**). The strength of this strategy is that the substituents at the two Dap positions are identified in a fashion that ensures they are able to cooperatively engage the SH2 domain. However, this strategy possesses the disadvantage that if multiple leads are observed from the initial library (**18**) then multiple sublibraries of **20** must be prepared. The "parallel" synthetic strategy (Scheme 3b) circumvents the issue of possible multiple sublibraries by offering a convergent route to the final lead species **21**. Libraries at multiple sites are prepared in parallel (i.e., **18** and **22**) and simultaneously screened to identify lead substituents at the Dap residues (i.e., **19** and **23**). Multiple lead substituents at these sites are subsequently combined in a few final peptides, which are then directly assessed for affinity. The obvious weakness associated with the parallel strategy is that the substituents at the two Dap moieties are identified independently of each other. Consequently, it is possible that the lead substituents at the different Dap sites might require the peptide to assume vastly different, and therefore incompatible, conformations when bound to the SH2 surface. However, in the specific case of the pTyr-Xaa-Xaa-Ile-/Lck SH2 domain complex, structural studies suggest that the bound peptide is unlikely to display significant conformational flexibility.[20] Therefore, we employed the Scheme 3b strategy and obtained multiple lead substituents at the two Dap sites. Individual peptides containing various combinations of these substituents were prepared, and their efficacy as Lck SH2 ligands was assessed. The lead doubly Dap-modified species **24** possesses an affinity for the Lck SH2 domain of 200 pM, approximately 4 orders of magnitude better than the starting consensus peptide acetyl-pTyr-

Scheme 3



Glu-Glu-Ile-amide. To the best of our knowledge, compound **24** is the tightest binding SH2-directed ligand ever reported.

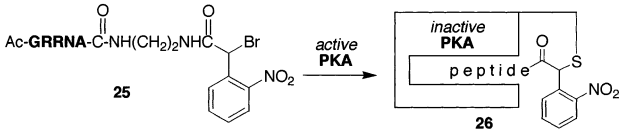


We have also applied the strategy described above to various PKC isoforms. In this case, the Scheme 3a iterative strategy was employed, which furnished a PKCα inhibitor that is 3 orders of magnitude more potent than any previously reported peptide-based inhibitor for this enzyme.[21] Finally, in collaboration with my colleague Zhong-Yin Zhang, we developed a variant of the approach outlined in Scheme 2 to identify high-affinity inhibitors for PTP1B, a protein phosphatase that serves as a negative regulator of insulin action. Previous attempts to apply a "rational" design and synthesis strategy for the preparation and identification of PTP1B inhibitors resulted in species displaying modest inhibitory activities in the low micromolar range.[22] However, a library-based methodology analogous to the Scheme 2 strategy furnished a PTP1B inhibitor that exhibits a more impressive 2.4 nM K_i.[23] Furthermore, this inhibitor displays extraordinary selectivity for PTP1B relative to a host of other protein phosphatases.

Photoactivation and Inhibition of Signal Transduction Pathways

The response of a cell to a stimulus is controlled by which signaling pathways are activated as well as the precise timing of their activation. The introduction of temporal issues makes the complexities associated with assessing the role of specific protein kinases in signal transduction even more challenging. Fortunately, exquisite temporal control is possible by the use of functionally inert ("caged") compounds whose activities can be rapidly unleashed upon exposure to a strong light source.[24,25] A number of caged messenger compounds have been described, including caged NO, cAMP, and Ca^{2+}. However, until recently, temporal control over individual protein members of signaling pathways was unknown.

PKA is the first protein kinase to have its three-dimensional structure determined.[26] Furthermore, the cellular phenotype associated with PKA activation is well documented.[27] This information was crucial for the preparation and subsequent intracellular evaluation of a photoactivatable PKA. The affinity label **25** was designed so that the reactive benzyl bromide moiety is positioned adjacent to the active site cysteine of PKA when enzyme bound (cf. **9**).[28] Key structural elements contained within



25 include (1) an electrophilic site that, upon attachment to the sulfhydryl of Cys-199, creates a photocleavable locus between the peptide and the enzyme and (2) an amino acid sequence that furnishes only modest affinity for the

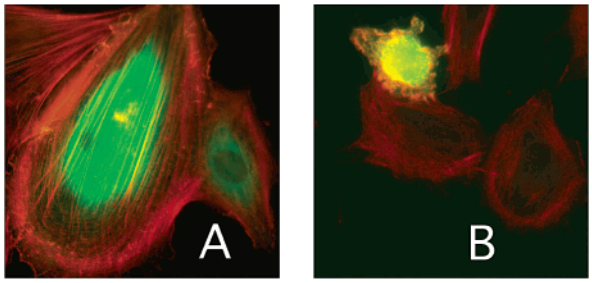


FIGURE 1. Overlay images of rhodamine-phalloidin staining for F-actin (red) and FITC-IgG staining for microinjected cells (green) in (a) REFs microinjected with caged PKA but not photochemically activated and (b) REFs microinjected with caged PKA and photoactivated.

protein kinase. The latter structural feature ensures that once the peptide is photocleaved from the enzyme, it will be unable to serve as a reversible inhibitor of the enzyme. Exposure of PKA to affinity label **25** provided the modified kinase **26**, which has less than 2% of the activity displayed by the native enzyme. Photolysis of **26** regenerates kinase activity to a level that is 50% of that of the native enzyme. Hagan Bayley and his colleagues have also described the preparation of a caged PKA but employed a nonpeptide-based strategy to modify Cys-199.[29]

Previous studies have demonstrated that activated PKA will generate profound changes in cellular morphology, including a loss of stress fibers, a rounding-up of cellular shape, and ruffling of the cellular membrane.[27] Microinjection of caged PKA **26** into rat embryo fibroblasts (REF-microinjected cells are stained green) in the absence of light fails to elicit any of these responses (Figure 1a). By contrast, microinjection followed by photolysis leads to the expected morphological changes (Figure 1b). Furthermore, adjacent nonmicroinjected cells (red), although exposed to photolysis, do not display any characteristics of an activated PKA pathway (i.e., they retain their stress fibers, their elongated shape, and their smooth membrane surface).

Although this work demonstrates that intracellular signaling cascades can be light-initiated at distinct protein kinase-occupied points along the cascade, in a very real sense PKA represents an unusually simple example of protein kinase-mediated signaling. PKA activity is intracellularly controlled by the reversible formation of an inactive complex between catalytic and regulatory subunits. Since the intracellular ratio of catalytic and regulatory subunits is approximately 1:1, direct microinjection of caged catalytic subunit (and subsequent uncaging) generates excess catalytic subunit. By contrast, the activity of many signaling proteins, including many protein kinases, is controlled by phosphorylation. Under these circumstances, photouncaging a protein kinase might lead to only transient or even negligible activation of a signaling pathway, since the light-activated enzyme could be immediately switched off via phosphorylation (or dephosphorylation) catalyzed by endogenous enzymes. In short, the design of any photoactivatable protein must be mindful of intracellular control mechanisms that could

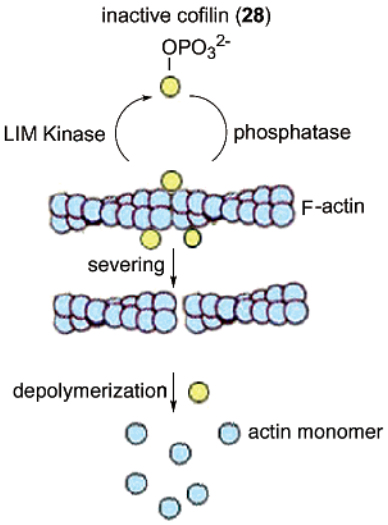
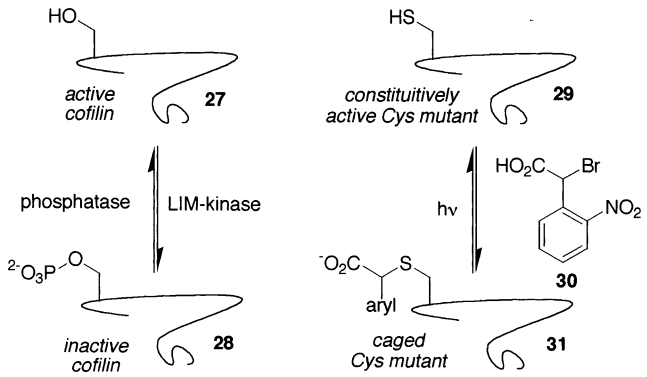


FIGURE 2. Mechanism of severing and depolymerization of F-actin by cofilin.

Scheme 4



immediately inactivate the light-uncaged enzyme. A case in point is illustrated in Figure 2. Epidermal growth factor (EGF)-stimulated cell motility is mediated by the cofilin-driven cleavage of existing filamentous-actin (F-actin). The active form of cofilin (**27**) contains a free serine at position 3 (Scheme 4). Phosphorylation at this site by LIM-kinase generates the inactive cofilin **28**. Consequently, the design of any caged cofilin must not only take into account how to create a light-activatable form of the protein, but must also circumvent the intracellular mechanism by which cofilin activity is endogenously controlled.

In collaboration with my colleague John Condeelis, we prepared the caged cofilin (**31**).[30] A constitutively active cofilin was first generated, a species that could not be phosphorylated by LIM-kinase, its' negative regulator. We had previously found that cysteine residues, when incorporated into the serine position of protein kinase peptide substrates, fail to undergo phosphorylation.[31] The serine-to-cysteine mutant **29** displays high F-actin severing activity and is resistant to LIM-kinase-catalyzed phosphorylation. The cysteine moiety in **29** has the additional attribute that it is easily modifiable. The cofilin mutant, upon reaction with **30** furnishes **31**, a species that electrostatically resembles the inactive phosphorylated wild-type enzyme **28** (Scheme 4). Indeed, caged cofilin

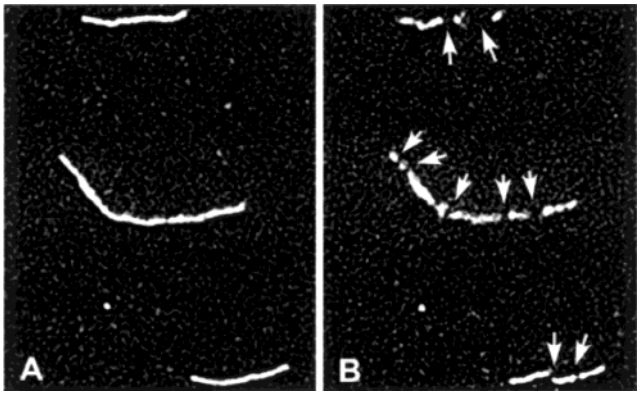


FIGURE 3. (A) Rhodamine-labeled F-actin filaments (B) in the presence of irradiated caged Cys-3 cofilin **31**. Cleavage sites along the F-actin filaments are marked with arrows.

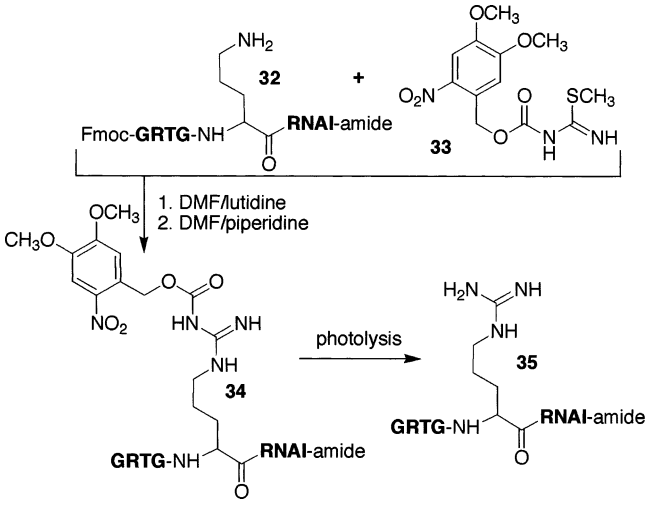
31 is not able to depolymerize F-actin. Upon exposure to high-intensity light, approximately 80% of Cys-3 cofilin **29** activity is restored. The action of caged cofilin, both before and after illumination, is visually obvious in Figure 3. F-actin can be directly observed by copolymerizing rhodamine-actin along with native and biotin-labeled actin. Actin filaments are then simply attached to a nitrocellulose coated microscope slide using an anti-biotin antibody. The various forms of cofilin can be perfused into the slide chamber, and severing of the fluorescently labeled filaments can then be visualized under the microscope. Figure 3a shows the rhodamine-labeled filaments prior to introduction of cofilin. Multiple cleavage sites in these filaments are apparent following introduction of the photoirradiated caged cofilin **31**.

Caged signaling proteins, such as those described above, can be used to intracellularly activate signaling cascades at specific points along the pathway. This can furnish a direct assessment of the role played by a specific signaling protein in driving an observable phenotype. The clear advantage associated with these caged species is that the desired activity can be switched on whenever or wherever the experimenter so desires. In addition to photoinitiating a signaling cascade with caged signaling proteins, it is also possible to phototerminate a cascade with caged inhibitors. Along these lines, we conducted intracellular experiments analogous to those shown in Figure 3 with a photoactivatable PKA inhibitor. Key Arg residues in active site-directed PKA peptides are important for PKA recognition.[32,33] We developed the nitroveratroloxycarbonyl-based agent (**33**) which converts ornithine (Orn) residues into caged Arg moieties (Scheme 5).[34] Photoactivation of the caged **34** generates **35**, which intracellularly blocks PKA-driven changes in cell morphology.

Fluorescent Probes of Protein Kinase Activity

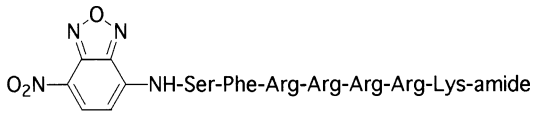
The issue of when protein kinases are activated in response to a stimulus has proven difficult to address. Lysis of synchronized cell populations followed by capture of the kinase in question and measurement of its activity furnishes some information concerning catalytic status,

Scheme 5



but under conditions that are nonphysiological in nature. By contrast, intracellular fluorometric probes of protein kinase activity offer the potential of real time assessment of signaling activity under physiologically relevant conditions.

We have prepared two distinct classes of phosphorylation-responsive fluorescent peptide substrates, both of which evolved from our observation that protein kinases catalyze the phosphorylation of synthetic substrates containing N- or C-terminus-substituted serine residues. The first class of fluorescent protein kinase substrate probes was based upon the simple notion that the photophysical properties of a fluorophore, directly positioned on the serine residue within a few angstroms of the phosphorylatable alcohol, should undergo a dramatic change in response to phosphorylation. We prepared a library of >400 compounds of the structure fluorophore-Ser-Phe-Arg-Arg-Arg-Arg-Lys-amide, which contains an amino acid sequence recognized by the PKC isoforms α, β, and γ.[35] Peptide **36** was identified as the lead substrate from this library. Not only does **36** display a phosphorylation-



36 PKCα K_m = 31 μM; V_{max} = 2.3 μmol/min-mg

induced change in fluorescence that is 1 order magnitude greater than what had been previously observed for any fluorophore-appended peptide-based protein kinase substrate,[35] it also serves as an outstanding substrate for the PKC α, β, and γ isoforms.

PKCs are activated during mitosis.[36] Consequently, we examined the ability of peptide **36** to report PKC activity in mitotic lysates from HeLa cells. Crude mitotic cell extracts were prepared from nocodazole-synchronized cells and the PKC assay initiated via addition of the cell lysate to an assay buffer containing **36**. A linear increase in fluorescence intensity is observed during the first 10 min following addition of cell lysate, which then

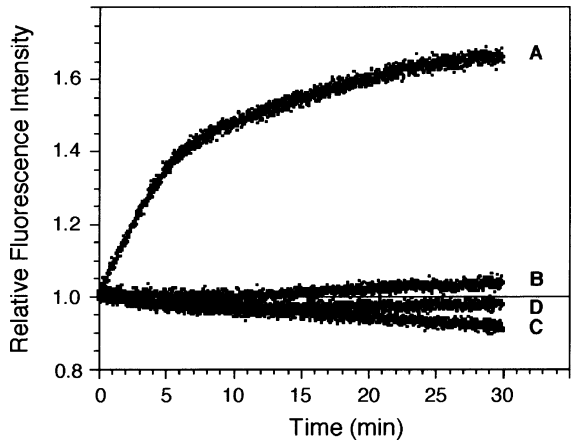
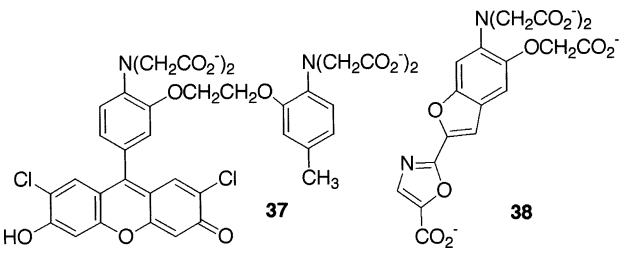


FIGURE 4. PKC activity in mitotic HeLa cell lysates. Fluorescence change as a function of incubation time (A) in the presence of mitotic cell lysate, (B) in the absence of cell lysate, (C) in the presence of cell lysate and staurosporine, and (D) in the presence of PKC immunodepleted cell lysate. Figure 4 is reproduced with permission from the American Society for Biochemistry and Molecular Biology (copyright 2002).

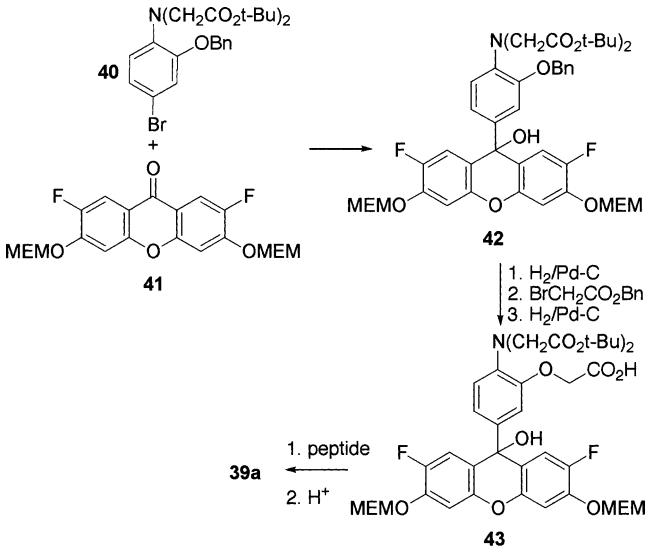
plateaus at approximately 1.7-fold above background (Figure 4, curve **A**). Staurosporine blocks the increase in fluorescence intensity observed upon mitotic cell lysate addition (Figure 4, curve **C**). However, staurosporine[37] targets a variety of protein kinases. Consequently, the staurosporine-induced block of fluorescence could be due to inhibition of PKC activity or the inhibition of other protein kinases that also catalyze the phosphorylation of peptide **36**. We addressed whether **36** is selective for the PKC by immunodepleting the mitotic cell lysates of PKC α, β, and γ. The PKC-depleted lysate fails to elicit a change in fluorescence intensity (Figure 4, plot **D**), an observation consistent with the notion that the fluorescence enhancement observed with crude mitotic lysates is due to PKC activity.

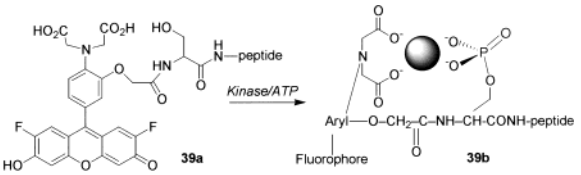
Our second class of fluorophore-responsive protein kinase peptide substrates was inspired by the Ca^{2+}-sensitive fluorophores (e.g., **37**) described by Tsien and co-workers.[38,39] Formation of the Ca^{2+}-fluorophore complex, via coordination to all 4 carboxylates, is manifested by a dramatic fluorescence change. Ca^{2+} coordination induces a twist about the bond between the amine and the chromophore, altering the electronic interaction between the nitrogen lone pair and the fluorescent ring system, thereby leading to a change in fluorescence. A Mg^{2+} chelator, based upon the same principles, has also been described (**38**).[40]



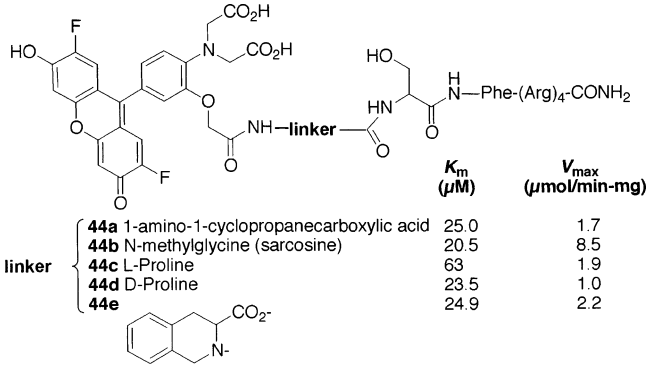
We designed several peptide-based species that contain some of the structural features present in Tsien's compounds (e.g., **39a**).[41] The M^{2+} receptor site generated upon phosphorylation (**39b**) is comprised of two carboxylates and the newly introduced phosphate moiety. Upon divalent metal ion coordination, a fluorescence enhancement should transpire via a mechanism analogous to the one proposed for **37** and **38**. Compound **39a** was prepared via the protocol outlined in Scheme 6. PKCα-catalyzed phosphorylation of **39a** (where peptide = Phe-Arg-Arg-Arg-Arg-amide) generates **39b**, which displays a 140% increase in fluorescence ($\lambda_{excitation}$ = 488 nm; $\lambda_{emission}$ = 523 nm). However, although the K_m value (26.5 M) for the PKCα-catalyzed phosphorylation of **39a** is very good, the corresponding V_{max} (0.32 mol min^{-1} mg^{-1}) is disappointing. One possible explanation for the latter is the close proximity of the fluorophore to the phosphorylatable serine, which may negatively impact the phosphorylation rate. We addressed this possibility by preparing a small library of 22 derivatives of **39** using the disulfide-based resin **13** (Scheme 2). All members of this library contain a different turn-promoting linker between the peptide and the fluorophore. Our rationale was that although the large fluorophore in derivatives **44** lies at a larger through-bond distance from the site of phosphorylation, the turn-inducing nature of the linkers should enable the iminodiacetic acid carboxylates and newly introduced phosphate to assume a position that promotes metal chelation following phosphorylation. Five members of this library display a significant enhancement in fluorescence upon exposure to PKCα and ATP [(**44a**) 164%, (**44b**) 264%, (**44c**) 156%, (**44d**) 150%, (**44e**) 167%)]. The K_m and V_{max} values are quite favorable, particularly for the sarcosine analogue **44b**.

Scheme 6





In our initial cell-based studies, we employed the lead fluorophore-appended peptide substrate (**36**) identified



from the 400-member fluorophore-Ser-Phe-Arg-Arg-Arg-Arg-Lys-amide library. The HeLa cell line containing microinjected **36** was exposed to phorbol ester, a PKC activator. There is an obvious and dramatic change in fluorescence intensity within minutes following phorbol ester addition (Figure 5; and see the quicktime video available online). The curves generated in the lysate (Figure 4) and live cell assays[35] (not shown) are remarkably similar, displaying a linear increase in fluorescence intensity following the first 8−10 min of exposure to phorbol ester. Furthermore, the phorbol ester-induced change in fluorescence intensity displayed by peptide **36** in living cells is 2-fold, whereas the cell lysate-based experiments furnished an overall 1.7-fold change in fluorescence intensity. We also found that the PKC inhibitor, GF 109203X, effectively blocks the phorbol ester-induced enhancement in cellular fluorescence. Although the K_i value for GF 109203X is in the low nanomolar range, micromolar concentrations of GF 109203X were used to block in vivo PKC activity due to the high intracellular concentration of ATP.[16]

Conclusion

DNA microarrays have been used to sample time-dependent changes in gene expression patterns in response to environmental stimuli.[42] Analogous methods are under development to assess changes in protein levels and/or activities.[43] However, activity sampling is commonly performed under decidedly noncellular conditions, which raises legitimate questions concerning the relevance of the acquired data to the intracellular state. The work underway in our laboratory has focused on the development of molecular tools to probe the role of individual protein kinases in cell-based phenotypes under physiological conditions. The issue of inhibitor and substrate specificity has received enormous attention given the large number of protein kinases encoded by the human genome. The majority of inhibitors described to date target the ATP binding site. By contrast, consensus sequence peptides have received comparatively little attention due, in large part, to their low binding affinity and apparent low specificity. The library-based strategy outlined in Scheme 2 offers one approach to identify selective, high-affinity substrates and inhibitors for an array of signaling proteins. Although protein kinase-selective/specific inhibitors and substrates have served as valuable reagents in signal transduction research, these reagents are unable to address the temporal relationships that exist between cellular phenotype and the activation of specific signaling proteins. In this regard, the clear advantage associated with caged proteins (and their corresponding inhibitors) is that the desired activity can be switched on (or off) whenever or wherever the experimenter so desires. Finally, fluorescent probes of protein kinase activity provide a visual intracellular read-out of when (and potentially where) protein kinases are activated in response to an extra- or intracellular stimulus. The latter provides an entry into examining the spatiotemporal dynamics of protein kinase action in the exciting field of protein kinase-mediated signal transduction.

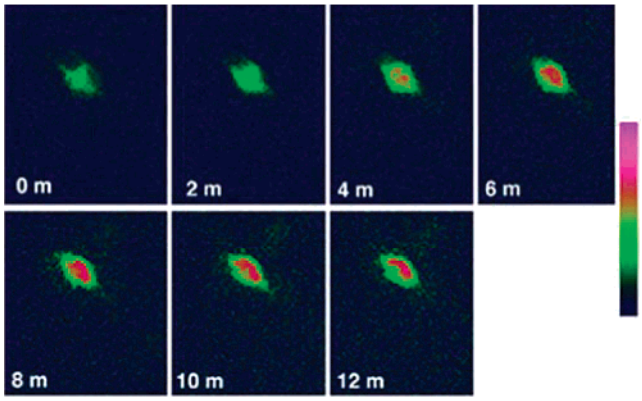


FIGURE 5. Time-lapse images of TPA-stimulated HeLa cells microinjected with peptide **36**.

Ⓦ These images serve as a link to a quicktime video available online which shows four cells, microinjected with peptide **36**, responding to a TPA stimulus. Both Figure 5 and the quicktime video are reproduced with permission from the American Society for Biochemistry and Molecular Biology (copyright 2002).

I am pleased to acknowledge the experimental and intellectual contributions of my co-workers and the generous support provided by the National Institutes of Health.

References

(1) Zhou, S.; Cantley, L. C. Recognition and Specificity in Protein Tyrosine Kinase-Mediated Signaling. *Trends Biochem. Sci.* **1995**, *20*, 470−475.
(2) Kwon, Y. G.; Mendelow, M.; Srinivasan, J.; Lee, T. R.; Pluskey, S.; Salerno, A.; Lawrence, D. S. The Active Site Substrate Specificity of the cAMP-Dependent Protein Kinase. *J. Biol. Chem.* **1993**, *268*, 10713−10716.
(3) Yan, X.; Curley, K.; Lawrence, D. S. The Specificity of the Protein Kinase C Alpha, Beta and Gamma Isoforms as Assessed by an Unnatural Alcohol-Appended Peptide Library. *Biochem. J.* **2000**, *349*, 709−715.
(4) Kwon, Y. G.; Srinivasan, J.; Mendelow, M.; Pluskey, S.; Lawrence, D. S. The cAMP-Dependent Protein Kinase Discriminates Between Prochiral Hydroxyl Groups. *J. Am. Chem. Soc.* **1993**, *115*, 7525−7528.
(5) Wood, J. S.; Yan, X.; Mendelow, M.; Corbin, J. D.; Francis, S. H.; Lawrence, D. S. Precision Substrate Targeting of Protein Kinases. The cGMP- and cAMP-Dependent Protein Kinases. *J. Biol. Chem.* **1996**, *271*, 174−179.
(6) Kwon, Y. G.; Mendelow, M.; Lawrence, D. S. The Active Site Substrate Specificity of Protein Kinase C. *J. Biol. Chem.* **1994**, *269*, 4839−4844.
(7) Yan, X.; Lawrence, D. S.; Corbin, J. D.; Francis, S. H. Distinguishing Between A Mitogenic and Two Closely Related Nonmitogenic Protein Kinases. *J. Am. Chem. Soc.* **1996**, *118*, 11684−11685.

(8) Yan, X.; Corbin, J. D.; Francis, S. H.; Lawrence, D. S. Precision Targeting of Protein Kinases. An Affinity Label that Inactivates the cGMP- but not the cAMP-Dependent Protein Kinase. *J. Biol. Chem.* **1996**, *271*, 1845−1848.
(9) Lev-Ram, V.; Jiang, T.; Wood, J.; Lawrence, D. S.; Tsien, R. Y. Synergies and Coincidence Requirements Between NO, cGMP, and Ca^{2+} in the Induction of Cerebellar Long-Term Depression. *Neuron* **1997**, *18*, 1025−1038.
(10) Dostmann and his colleagues recently constructed an extraordinarily potent 24 amino acid-containing PKG selective inhibitor; see Dostmann, W. R.; Taylor, M. S.; Nickl, C. K.; Brayden, J. E.; Frank, R.; Tegge, W. J. Highly Specific, Membrane-Permeant Peptide Blockers of cGMP-Dependent Protein Kinase Ialpha Inhibit NO−Induced Cerebral Dilation. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 14772−7.
(11) Arancio, O.; Antonova, I.; Gambaryan, S.; Lohmann, S. M.; Wood, J. S.; Lawrence, D. S.; Hawkins, R. D. Presynaptic Role of cGMP-Dependent Protein Kinase During Long-Lasting Potentiation. *J. Neurosci.* **2001**, *21*, 143−149.
(12) Lee, T. R.; Till, J. H.; Lawrence, D. S.; Miller, W. T. Precision Substrate Targeting of Protein Kinases v-Abl and c-Src. *J. Biol. Chem.* **1995**, *270*, 27022−6.
(13) Lee, T. R.; Niu, J.; Lawrence, D. S. The Extraordinary Active Site Substrate Specificity of $pp60^{c-src}$. A Multiple Specificity Protein Kinase. *J. Biol. Chem.* **1995**, *270*, 5375−80.
(14) Niu, J.; Lawrence, D. S. L-Dopa: A Potent Nonphosphorylatable Tyrosine Mimetic for $pp60^{c-src}$. *J. Am. Chem. Soc.* **1997**, *119*, 3844−3845.
(15) Niu, J.; Lawrence, D. S. Nonphosphorylatable Tyrosine Surrogates. Implications for Protein Kinase Inhibitor Design. *J. Biol. Chem.* **1997**, *272*, 1493−9.
(16) Lawrence, D. S.; Niu, J. Protein Kinase Inhibitors: The Tyrosine-Specific Protein Kinases. *Pharmacol. Ther.* **1998**, *77*, 81−114.
(17) Maly, D. J.; Choong, I. C.; Ellman, J. A. Combinatorial Target-Guided Ligand Assembly: Identification of Potent Subtype-Selective c-Src Inhibitors. *Proc Natl Acad Sci U S A* **2000**, *97*, 2419−24.
(18) Yeh, R. H.; Lee, T. R.; Lawrence, D. S. From Consensus Sequence Peptide to High Affinity Ligand, A "Library Scan" Strategy. *J. Biol. Chem.* **2001**, *276*, 12235−40.
(19) Lee, T. R.; Lawrence, D. S. Acquisition of High-affinity, SH2−Targeted Ligands via a Spatially Focused Library. *J. Med. Chem.* **1999**, *42*, 784−7.
(20) Kuriyan, J.; Cowburn, D. Modular Peptide Recognition Domains in Eukaryotic Signaling. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 259−288.
(21) Lee, J.-H.; Nandy, S. K.; Lawrence, D. S. Unpublished results, 2002.
(22) Taing, M.; Keng, Y.-F.; Shen, K.; Lawrence, D. S.; Zhang, Z.-Y. Potent and Highly Selective Inhibitors of the PTP1B Protein Tyrosine Phosphatase. *Biochemistry* **1999**, *38*, 3793−3803.
(23) Shen, K.; Keng, Y. F.; Wu, L.; Guo, X. L.; Lawrence, D. S.; Zhang, Z. Y. Acquisition of a Specific and Potent PTP1B Inhibitor from a Novel Combinatorial Library and Screening Procedure. *J. Biol. Chem.* **2001**, *276*, 47311−47319.
(24) Adams, S. R.; Tsien, R. Y. Controlling Cell Chemistry with Caged Compounds. *Annu. Rev. Physiol.* **1993**, *55*, 755−784.
(25) McCray, J. A.; Trentham, D. R. Properties and Uses of Photoreactive Caged Compounds. *Annu. Rev. Biophys. Biophys. Chem.* **1989**, *18*, 239−270.
(26) Smith, C. M.; Radzio-Andzelm, E.; Madhusudan; Akamine, P.; Taylor, S. S. The Catalytic Subunit of cAMP-Dependent Protein Kinase: Prototype for an Extended Network of Communication. *Prog. Biophys. Mol. Biol.* **1999**, *71*, 313−41.
(27) Roger, P. P.; Rickaert, F.; Huez, G.; Authelet, M.; Hofmann, F.; Dumont, J. E. Microinjection of Catalytic Subunit of Cyclic AMP-Dependent Protein Kinase Triggers Acute Morphological Changes in Thyroid Epithelial Cells. *FEBS Lett.* **1988**, *232*, 409−413.
(28) Curley, K.; Lawrence, D. S. Photoactivation of a Signal Transduction Pathway in Living Cells. *J. Am. Chem. Soc.* **1998**, *120*, 8573−8574.
(29) Chang, C.; Fernandez, T.; Panchal, R.; Bayley, H. Caged Catalytic Subunit of cAMP-Dependent Protein Kinase. *J. Am. Chem. Soc.* **1998**, *120*, 7661−7662.
(30) Ghosh, M.; Ichetovkin, I.; Song, X.; Condeelis, J. S.; Lawrence, D. S. Light-Induced F−Actin Severing Via A Caged Cofilin. *J. Am. Chem. Soc.* **2002**, *124*, 2440−2441.
(31) Prorok, M. F.; Lawrence, D. S. Unpublished results, 1987.
(32) Scott, J. D.; Glaccum, M. B.; Fischer, E. H.; Krebs, E. G. Primary-Structure Requirements for Inhibition by the Heat-Stable Inhibitor of the cAMP-Dependent Protein Kinase. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1613−1616.
(33) Glass, D. B.; Cheng, H. C.; Mende-Mueller, L.; Reed, J.; Walsh, D. A. Primary Structural Determinants Essential for Potent Inhibition of cAMP-Dependent Protein Kinase by Inhibitory Peptides Corresponding to the Active Portion of the Heat-Stable Inhibitor Protein. *J. Biol. Chem.* **1989**, *264*, 8802−88010.
(34) Wood, J.; Koszelak, M.; Liu, J.; Lawrence, D. S. A Caged Protein Kinase Inhibitor. *J. Am. Chem. Soc.* **1998**, *120*, 7145−7146.
(35) Yeh, R. H.; Yan, X.; Cammer, M.; Bresnick, A. R.; Lawrence, D. S. Real Time Visualization of Protein Kinase Activity in Living Cells. *J. Biol. Chem.* **2002**, *277*, 11527−11532.
(36) Varlamova, O.; Spektor, A.; Bresnick, A. R. Protein Kinase C Mediates Phosphorylation of the Regulatory Light Chain of Myosin-II During Mitosis. *J. Muscle Res. Cell Motil.* **2001**, *22*, 214−250.
(37) Way, K. J.; Chou, E.; King, G. L. Identification of PKC−Isoform-Specific Biological Actions Using Pharmacological Approaches. *Trends Pharmacol. Sci.* **2000**, *21*, 181−187.
(38) Kao, J. P.; Harootunian, A. T.; Tsien, R. Y. Photochemically Generated Cytosolic Calcium Pulses and Their Detection by Fluo-3. *J. Biol. Chem.* **1989**, *264*, 8179−8184.
(39) Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. A New Generation of Ca^{2+} Indicators with Greatly Improved Fluorescence Properties. *J. Biol. Chem.* **1985**, *260*, 3440−3450.
(40) Raju, B.; Murphy, E.; Levy, L. A.; Hall, R. D.; London, R. E. A Fluorescent Indicator for Measuring Cytosolic Free Magnesium. *Am. J. Physiol.* **1989**, *256*, C540−548.
(41) Chen, C.-A.; Yeh, R. H.; Lawrence, D. S. Design and Synthesis of a Fluorescent Reporter of Protein Kinase Activity. *J. Am. Chem. Soc.* **2002**, *124*, 3840−3841.
(42) Blohm, D. H.; Guiseppe-Elie, A. New developments in microarray technology. *Curr. Opin. Biotech.* **2001**, *12*, 41−7.
(43) Fung, E. T.; Thulasiraman, V.; Weinberger, S. R.; Dalmasso, E. A. Protein biochips for differential profiling. *Curr. Opin. Biotech.* **2001**, *12*, 65−9.

AR020132S