Identification of Novel Classes of Protein Kinase Inhibitors Using Combinatorial Peptide Chemistry Based on Functional Genomics Knowledge

Thomas J. Lukas, Salida Mirzoeva, Urszula Slomczynska, and D. Martin Watterson*

Drug Discovery Program and Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, Illinois 60611

Received October 8, 1998

A discovery approach based on an intramolecular inhibitory mechanism was applied to a prototype calmodulin (CaM)-regulated protein kinase in order to demonstrate a proof-of-principle for the development of selective inhibitors. The overall approach used functional genomics analysis of myosin light chain kinase (MLCK) to identify short autoinhibitory sequences that lack CaM recognition activity, followed by recursive combinatorial peptide library production and comparative activity screens. Peptide **18** (Arg-Lys-Lys-Tyr-Lys-Tyr-Arg-Arg-Lys-NH_2), one of several selective inhibitors discovered, has an $IC_{50} = 50$ nM for MLCK, inhibits CaM kinase II only at 4000-fold higher concentrations, and does not inhibit cyclic AMP-dependent protein kinase. Analogues of peptide **18** containing conformationally constrained *cis*-4-aminocyclohexanecarboxylic acid retained affinity and selectivity. The inhibitors add to the armamentarium available for the deconvolution of complex signal transduction pathways and their relationship to homeostasis and disease, and the approach is potentially applicable to enzymes in which the catalytic and regulatory domains are found within the same open reading frame of a cDNA.

Introduction

Ca^{2+} is key to the regulation of a diverse array of cellular pathways, and intracellular Ca^{2+} signal transduction pathways are attractive in the search for potential new drug discovery targets. For example, clinically effective drugs that alter intracellular Ca^{2+} signal transduction pathways are available, but their therapeutic index and untoward effects are a limitation to their broader use.[1,2] The problems with such calcium antagonist drugs are not surprising based on the knowledge that intracellular Ca^{2+} signaling is complex, with specificity primarily in the downstream components of the pathways. For example, the first molecular discrimination is the calcium receptor protein, with calmodulin (CaM) being the most extensively characterized.[3] However, CaM itself is not an attractive drug discovery target because it functions as a Ca^{2+} binding subunit of approximately a dozen different classes of enzymes. The most detailed knowledge about CaM-regulated enzymes is that for the CaM-regulated serine/threonine protein kinases, of which there are at least 10 groups.[4] Each CaM-regulated protein kinase controls distinct cellular response pathways. It is at this downstream point in the CaM-mediated Ca^{2+} signal transduction pathways, i.e., at the level of the CaM-regulated protein kinases, that there is a degree of control that is attractive for investigations on ligand modulation of biological responses. There is increased biological specificity due to the selection of CaM as the calcium receptor and the inherent properties of each CaM-regulated protein kinase, such as peptide substrate recognition and cellular localization. In addition, this is a point in the pathway where stoichiometric interactions, such as the recognition of the Ca^{2+} signal by CaM, are amplified due to the catalytic properties of the kinase. Additional amplification may occur if the kinase substrate, in turn, influences the activity of other downstream enzymes. Because of the biological selectivity and signal amplification potential at this point in the Ca^{2+} signal transduction mechanism and the demonstration that serine/threonine protein kinases can be attractive drug discovery targets,[5,6] the CaM-regulated protein kinases should be investigated as potential targets for ligand modulation of biological responses.

The development of selective kinase inhibitors is needed for the further dissection of signal transduction pathways and their links to biological responses. However, specific compounds that might prove useful in such critical biological investigations of CaM-regulated pathways are lacking, and the database of available inhibitors has not provided a good starting point for the development of drugs or lead compounds. Although inhibitors targeted to the ATP substrate site of serine/threonine protein kinases have proven valuable in the dissection of molecular mechanisms[7] and have served as lead compounds for drug development,[5] CaM-regulated protein kinase inhibitors that compete with the ATP substrate lack the desired biological and biochemical selectivity.[8−11] In addition, the various CaM-regulated protein kinases overlap in terms of their ATP and peptide substrate recognition properties, adding to the problems with using only a substrate analogue approach as a starting point in the development of new classes of inhibitors. In contrast, an accumulating body of evidence has revealed that each CaM-regulated protein kinase has a level of selectivity in its CaM regulatory domain,[4] raising the possibility for a new focus in the development of selective inhibitors for this class of enzymes.

* Corresponding author address: 303 E. Chicago Ave., Chicago, IL 60611-3008. Tel: 312-503-0656. Fax: 312-503-1300. E-mail: m-watterson@nwu.edu.

10.1021/jm980573a CCC: $18.00
Published on Web 02/24/1999

Much has been learned over the past decade about the molecular basis of CaM recognition and activation of protein kinases, especially for vertebrate nonmuscle and smooth muscle myosin light chain kinase (MLCK).[4] While it is widely accepted that there are CaM regulatory sequences that flank the core catalytic segment, there is controversy concerning the relationships between sequence segments and mechanism of CaM recognition and enzyme inhibition. The controversy surrounds whether the functionally coupled activities of CaM recognition and intramolecular inhibition are centered in distinct regions that allow for a general allosteric model of CaM regulation or are centered in a common pseudosubstrate region. The CaM binding region of MLCK was first identified by Lukas et al.,[12] who purified and characterized a high-affinity CaM binding sequence, termed the RS20 region, from digests of MLCK isolated from vertebrate tissues. DNA probes based on these amino acid sequences were used by Shoemaker et al.[13] to isolate cDNA clones and demonstrate, by site-directed mutagenesis and enzymology experiments, the RS20 CaM recognition region in the context of a fully active protein kinase sequence. In addition, Shoemaker et al.[13] defined the relationship of the RS20 region to contiguous autoinhibitory sequences (referred to as AM13 region) that are functionally coupled to, but distinct from, the CaM recognition sequences. The relief-of-autoinhibition model proposed by Shoemaker et al.[13] was a general functional model that allowed for allosteric regulation of protein kinase activity by CaM and other effectors. This model is supported by more recent site-directed mutagenesis and proteolysis studies of MLCK,[14] the homology of the MLCK regulatory segment to domains in the crystal structure of a related CaM kinase,[15] and the ability of synthetic peptides based on the autoinhibitory sequence to inhibit MLCK catalytic activity without binding to CaM.[16] The simpler pseudosubstrate model[17] is based upon the sequence similarity between a region of MLCK and the phosphorylation site in its substrate, myosin light chain. It is proposed that the pseudosubstrate sequence occupies the active site of the enzyme, and activation is through a competition between CaM binding and active site binding. The pseudosubstrate model is supported by the ability of synthetic peptides based on the pseudosubstrate sequence to inhibit MLCK[17,18] and by the ability of an engineered MLCK with this sequence replaced by a phosphorylation site to undergo autophosphorylation.[19] Regardless of which model better describes the biological mechanism, synthetic peptides based on the pseudosubstrate sequence bind to CaM as well as inhibit MLCK, resulting in a lack of selectivity among CaM-regulated enzymes.[18,20] Therefore, the use of sequences based on the functional genomics analyses of Shoemaker et al.[13] would appear to be a reasonable focus in using MLCK as a prototype to demonstrate that autoinhibitory sequences can be used as starting points in the discovery of selective inhibitors of CaM-regulated protein kinases.

The increasing availability of automated combinatorial peptide chemistry technologies[21] provides an opportunity to utilize peptide libraries, based on the amino acid sequences identified from functional genomics investigations, as a starting point to probe for new high-affinity and selective inhibitors. The work described in this report provides a proof-of-principle for using recursive combinatorial peptide libraries to identify new potent and selective peptide inhibitors based on such functional genomics knowledge about a prototype CaM-regulated protein kinase. The selection of an appropriate starting sequence based on functional genomics and the use of comparative activity screens for each peptide library were the key features of the approach described here that allowed success where previous attempts using only sequence similarity and substrate analogue approaches had failed. Further, the knowledge base derived from the comparative activity analyses provides a starting point for future investigations into how inhibitor structure is related to function, potentially enhancing the future identification of peptidomimetic compounds. Clearly, additional investigations with a variety of enzymes are required to explore the broader application of such approaches, but the protein kinase inhibitors produced as a result of the investigations add to an accumulating array of tools needed to understand the complex area of protein kinases and their relationship to biological homeostasis and disease.

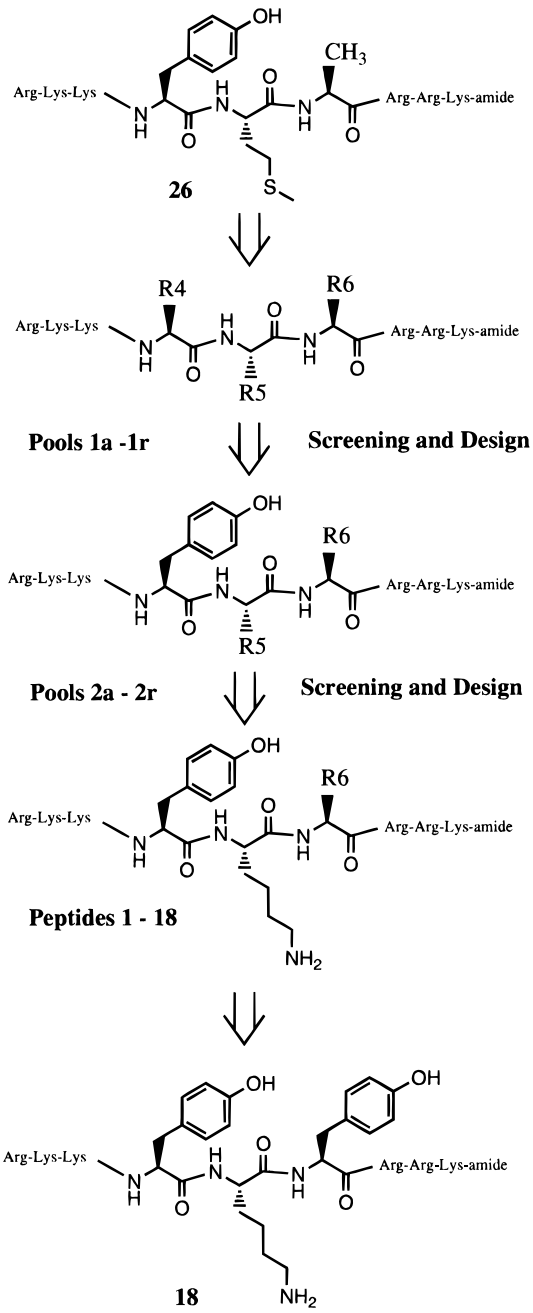
Results

Strategy and Rationale for Combinatorial Library Synthesis and Activity Screens. The combinatorial chemistry approach used in this study is based upon the sequential synthesis of peptide libraries as originally described by Houghten and co-workers.[21] In the original demonstration,[21] libraries were designed to allow deconvolution of the amino acid sequences in pools with the desired bacteriocidal activity. For our investigation, the activity analysis was for comparative protein kinase inhibitory properties using screening conditions with multiple enzyme targets that favor competition with peptide substrate rather than ATP. The recursive use and design of the libraries was based on the functional genomics studies, for the first library, and on the comparative activity analyses, for the second and third libraries.

The focus of the study was to improve the MLCK inhibitory activity of a lead peptide identified from functional genomics studies, while making the inhibition of CaM-dependent kinase II (CaMPKII) worse and maintaining the lack of cyclic AMP-dependent protein kinase (PKA) inhibition. Because PKA-mediated pathways undergo signal transduction cross-talk with CaM-mediated pathways,[4] it is imperative that inhibitors of CaM-regulated protein kinases do not inhibit PKA.

Selection of the lead peptide sequence for the focused library was based upon functional genomics and peptide analogue data for MLCK.[13,16] The core autoinhibitory sequence of MLCK was experimentally determined by site-directed mutagenesis, protein engineering, and enzymology.[13] The importance of the flanking basic amino acids in the starting nonapeptide sequence for autoinhibition for MLCK was demonstrated by the fact that mutation of multiple basic amino acids within the autoinhibitory segment renders the enzyme constitutively active.[13] The selectivity of the lead nonapeptide sequence for inhibition of MLCK was indicated by results with synthetic peptides, based upon the MLCK autoinhibitory segment, that showed competitive inhibi-

Scheme 1. Flow Diagram of Peptide Libraries Design and Deconvolution[a]



[a] Nonapeptide libraries based on the sequence of the lead peptide **26** (Arg-Lys-Lys-Tyr-Met-Ala-Arg-Arg-Lys-NH_2) were designed and screened to optimize the central tripeptide sequence toward MLCK inhibition and away from other related kinases. One of the best peptides for these criteria was found to be the peptide containing the Tyr-Lys-Tyr sequence at positions 4−6 (peptide **18**).

tion of peptide substrate phosphorylation and lack of CaM antagonism.[16] The lead nonapeptide, Arg-Lys-Lys-Tyr-Met-Ala-Arg-Arg-Lys-NH_2 (peptide **26**), inhibits MLCK (IC_{50} of 1.2 μM) and CaMPKII (IC_{50} = 90 μM), but not the catalytic subunit of PKA. Because of the need for the basic amino acids within the lead peptide sequence for selective inhibition of MLCK, the clusters of basic residues were not changed as part of the studies reported here. Therefore, the internal tripeptide segment was varied using amino acids with different side chains as shown in Scheme 1.

From the 20 naturally occurring amino acids, 18 were used for creating the combinatorial libraries. Cysteine and arginine were not used in the variable part of the sequence. Cysteine was not incorporated because of potential problems of oxidation/dimerization of the peptide. Arginine was not used because the inclusion of additional arginine to the core triad would add to the byproducts resulting from incomplete cleavage of the protecting group in peptides containing multiple arginine residues[22] and would limit the diversity of side chain properties in a lead peptide.

The recursive library design (Scheme 1) was one in which 18 pools of peptides were made with decreasing complexity based on the activity screen results. For example, the synthesis of the first library was designed to make 18 peptide pools in which there was a known amino acid at position 4 but all possible combinations of the 18 amino acids at positions 5 and 6. This results in 324 amino acid sequences being present in each pool of the first library, for a total analysis of 5832 possible amino acid sequences in the library. The inhibitory activity of all peptide pools was tested with the two closely related CaM-regulated protein kinases, MLCK and CaMPKII, and with PKA. On the basis of the activity screening results, the amino acid at position 4 (R4) was fixed for the next library production. The subsequent library would, thus, have 18-fold fewer sequences than the initial library. After selecting one or more residues for R5, the final library is a series of parallel syntheses in which the side chain R6 is varied in a set of 18 peptides that are purified and characterized.

Library Screening Results. The peptide pools from the first library (pools **1a**−**1r**) had IC_{50} values ranging from 0.18 to 14.2 μM for inhibition of MLCK. Peptide pools containing glutamic acid, glycine, or aspartic acid at position 4 were much less effective inhibitors of MLCK, suggesting that a negatively charged side chain or lack of appropriate conformational constraints at position 4 might diminish MLCK inhibitory activity, regardless of the neighboring functionality. Pools containing tyrosine, valine, or lysine at this position displayed improved inhibition compared to the lead peptide (Figure 1A), consistent with a potential need for conformational constraints that could be provided by chemically diverse side chains at position 4, in the context of several possible sequences found in the various combinations of amino acids at positions 5 and 6. Pool **1p** contains tyrosine at position 4, the same amino acid present in the lead peptide **26** and in the enzyme autoinhibitory segment, and has an IC_{50} = 0.18 μM. Pool **1p**, therefore, displays a 6.7-fold improvement in IC_{50} for MLCK compared to **26** and a 5-fold enhanced selectivity for MLCK inhibition against inhibition of CaMPKII (Figure 1B). Tyrosine was held constant at the 4-position in the next focused library based on the gain in selectivity and activity of a variety of potential sequences derived from the 18 possible amino acids at positions 5 and 6 and having tyrosine a position 4. It should be noted that, on the basis of the MLCK inhibitory activity, valine or lysine also could have been pursued further for a proof-of-principle investigation.

For the second library, 18 pools were made in parallel with position 5 being one of the possible 18 amino acids.

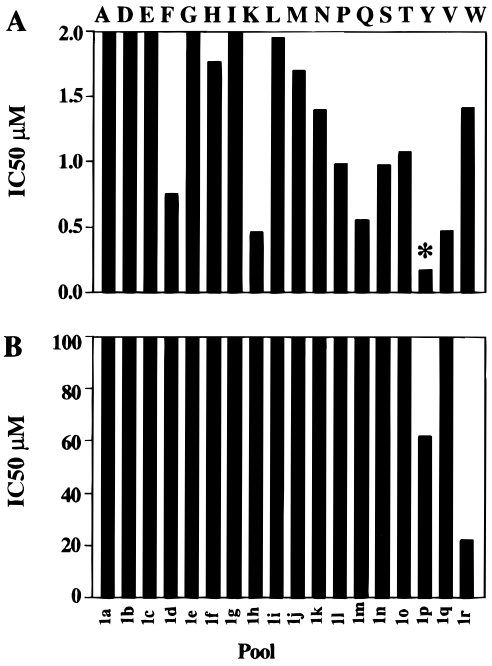


Figure 1. Summary of the inhibition of CaM-regulated protein kinases by peptide pools from the first combinatorial library. Pools from the library contained peptides where positions 5 and 6 are any of 18 amino acids chosen for combinatorial synthesis (see the text). Pools labeled **1a**−**1r** represent the presence at position 4 in the peptide of the amino acid Ala (**1a**), Asp (**1b**), Glu (**1c**), Phe (**1d**), Gly (**1e**), His (**1f**), Ile (**1g**), Lys (**1h**), Leu (**1i**), Met (**1j**), Asn (**1k**), Pro (**1l**), Gln (**1m**), Ser (**1n**), Thr (**1o**), Tyr (**1p**), Val (**1q**), or Trp (**1r**). Pool **1p** is marked with an asterisk; on the basis of the comparative activity of this pool, which has Tyr at the same position as the endogenous sequence found in peptide **26**, Tyr was fixed at position 4 in the design of the next library. IC_{50} values were estimated by a linear regression analysis on data points corresponding to 15−85% of maximal activity; here 100% is enzyme activity in the absence of inhibitors. The standard error of the IC_{50} values is 10% or less. A. Inhibition of MLCK. MLCK inhibition assays were done as described in the Experimental Section. The IC_{50} values for pools **1a**, **1b**, **1c**, **1e**, and **1g** are >2 μM. B. Inhibition of CaMPKII. CaMPKII inhibition assays were done as described in the Experimental Section. Pools with IC_{50} values shown as 100 μM on the graph lack inhibitory activity at this concentration.

At position 6, each pool was a mixture of the 18 amino acids. This resulted in 324 potential amino acid sequences in the library, arranged as 18 pools containing 18 possible amino acid sequences each. The pools from the second library (**2a**−**2r**) have IC_{50} values for MLCK inhibition spanning a 20-fold range from 0.09 to 1.79 μM (Figure 2A). All of the pools were relatively poor inhibitors of CaMPKII (Figure 2B). Only four pools were as good as or better than pool **1p**, the starting point from the first library. Pools containing Thr (**2o**) or Trp (**2r**) at position 5 had about the same IC_{50} for MLCK inhibition as pool **1p**. Pools containing lysine (**2h**) or valine (**2q**) at position 5 were about 1.5−2-fold better than pool **1p**, or 10−13-fold better than the starting peptide **26**. However, pool **2r** was not as attractive based on CaMPKII inhibition, and pools **2o**, **2r**, and **2q** were less attractive for further refinement than pool **2h** (lysine at position 5) when additional protein kinases, such as protein kinase C, were examined.[23] Pool **2h**, with R5 = $(CH_2)_4$-NH_2, provided >800-fold selectivity toward MLCK inhibition compared to CaMPKII. These

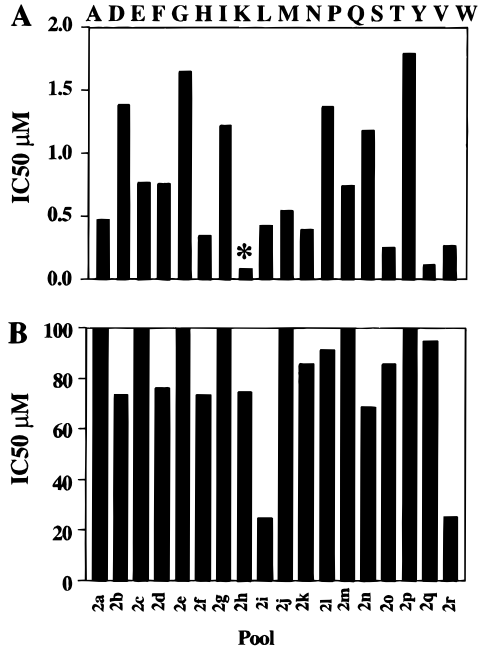


Figure 2. Summary of the inhibition of CaM-regulated protein kinases by peptide pools from the second combinatorial library. Pools from the library contained peptides where position 4 is tyrosine and position 6 is any of 18 amino acids chosen for combinatorial synthesis (see the text). Pools labeled **2a**−**2r** represent the presence at position 5 of the amino acid Ala (**2a**), Asp (**2b**), Glu (**2c**), Phe (**2d**), Gly (**2e**), His (**2f**), Ile (**2g**), Lys (**2h**), Leu (**2i**), Met (**2j**), Asn (**2k**), Pro (**2l**), Gln (**2m**), Ser (**2n**), Thr (**2o**), Tyr (**2p**), Val (**2q**), or Trp (**2r**). The most active and selective pool (**2h**) is marked with an asterisk. IC_{50} values were calculated from the data points corresponding to 15−85% of maximal activity by linear regression analysis. The standard error of the IC_{50} values is less than 10%. A. Inhibition of MLCK. MLCK inhibition assays were done as described in the Experimental Section. B. Inhibition of CaMPKII. CaMPKII inhibition assays were done as described in the Experimental Section. Pools with IC_{50} values displayed at 100 μM lack inhibitory activity at this concentration.

results suggested that when position 4 is a tyrosine, certain side chains at R5 contribute to the bias in favor of MLCK inhibition versus other protein kinases, although a clear trend was not apparent in terms of the side chain chemistry. As noted later, these observations raised the question of conformational restriction, rather than a particular side chain, as being a potential structure−function consideration.

To determine the more favorable side chains at position R6, parallel syntheses were done in which positions 4 and 5 were held constant (tyrosine and lysine, respectively) based on the results from the first two library screens. The amino acid at position 6 was varied with each of the possible 18 amino acids. The resulting 18 peptides were purified and characterized by amino acid analysis, HPLC, and mass spectrometry (Table 1). The IC_{50} values for MLCK inhibition ranged from 0.05 to 0.87 μM (Table 2). Peptides **4**, **7**, **10**, **12**, **14**, **17**, and **18** had the highest MLCK inhibition (IC_{50} ≤ 0.2 μM). These contain phenylalanine, isoleucine, methionine, proline, serine, tryptophan, or tyrosine at position 6. However, peptides **7** and **17** are less selective than the other peptides with respect to CaMPKII inhibition (Table 2). Five of the peptides (**4**, **10**, **12**, **14**, and **18**) exhibit >1000-fold selectivity as evaluated by the ratio of CaMPKII to MLCK inhibitory activity (Table

Table 1. Characterization of Peptides

peptide	sequence	amino acid composition[a]	MH^+ calcd	MH^+ found[b]
1	RKKYKARRK-NH_2	R(2.8), A(1.0), Y(1.0), K(4.1)	1233.6	1232.7
2	RKKYKDRRK-NH_2	R(2.9), D(1.0), Y(1.0), K(4.1)	1277.6	1277.2
3	RKKYKERRK-NH_2	R(2.9), E(1.1), Y(1.0), K(3.9)	1291.6	1291.1
4	RKKYKFRRK-NH_2	R(2.8), Y(1.0), F(1.0), K(4.1)	1309.7	1309.3
5	RKKYKGRRK-NH_2	R(2.6), G(0.9), Y(1.3), K(4.3)	1219.5	1219.0
6	RKKYKHRRK-NH_2	R(2.9), H(1.0), Y(1.1), K(4.1)	1299.6	1299.5
7	RKKYKIRRK-NH_2	R(3.0), I(0.9), Y(1.0), K(4.1)	1275.6	1275.2
8	RKKYKKRRK-NH_2	R(3.0), Y(0.9), K(5.0)	1290.7	1290.1
9	RKKYKLRRK-NH_2	R(2.8), L(1.2), Y(1.1), K(4.0)	1275.6	1274.4
10	RKKYKMRRK-NH_2	R(3.3), M(0.8), Y(1.0), K(4.0)	1293.7	1293.8
11	RKKYKNRRK-NH_2	R(3.0), Y(1.0), N(1.0), K(4.0)	1276.6	1276.2
12	RKKYKPRRK-NH_2	R(3.0), P(1.0), Y(1.0), K(4.0)	1259.6	1258.9
13	RKKYKQRRK-NH_2	R(2.9), Q(1.1), Y(0.9), K(4.0)	1290.6	1291
14	RKKYKSRRK-NH_2	R(3.0), S(0.9), Y(1.0), K(4.0)	1249.6	1250.1
15	RKKYKTRRK-NH_2	R(2.9), T(0.9), Y(1.0), K(4.1)	1263.6	1263.2
16	RKKYKVRRK-NH_2	R(3.1), V(0.9), Y(1.0), K(4.1)	1261.6	1261.7
17	RKKYKWRRK-NH_2	R(2.8), Y(0.9), K(4.3)	1348.7	1348.1
18	RKKYKYRRK-NH_2	R(3.0), Y(1.9), K(4.3)	1325.7	1324.3
19	RKKY(Ach)YRRK-NH_2[c]	R(3.1), Y(1.7), K(3.2), Ach(0.9)	1322.7	1323
20	RKK(Ach)KYRRK-NH_2	R(3.8), Y(1.1), K(3.2), Ach(1.1)	1287.7	1287.1
21	RKKYK(Ach)RRK-NH_2	R(3.1), Y(0.9), K(4.4), Ach(0.9)	1287.7	1287.7
22	RKKY(Ach)(Ach)RRK-NH_2	R(3.4), Y(0.8), K(3.6), Ach(1.5)	1284.6	1284.0
23	RKK(Ach)(Ach)YRRK-NH_2	R(3.3), Y(1.1), K(3.4), Ach(1.4)	1284.6	1284
24	RKK(Ach)K(Ach)RRK-NH_2	R(3.0), K(4.4), Ach(2.0)	1249.6	1249.0
25	RKK(Ach)(Ach)(Ach) RRK-NH_2	R(3.4), K(3.5), Ach(2.6)	1247.6	1246.6
26	RKKYMARRK-NH_2	R(2.8), A(1.1), Y(1.0), M(1.0), K(3.1)	1236.6	1236.1

[a] Amino acid analysis was done after acid hydrolysis, and amino acid ratios are normalized to the peptide molecular weight. [b] Protonated molecular ions were determined as described in the Experimental Section. [c] (Ach), *cis*-4-aminocyclohexanecarboxylic acid.

Table 2. Specificity of MLCK Inhibition by Peptides **1**−**18**

peptide	sequence	IC_{50}, μM[a] MLCK	IC_{50}, μM[a] CaMPKII	ratio IC_{50}(CaMPKII)/ IC_{50}(MLCK)
1	RKKYKARRK-NH_2	0.42	>200	>476
2	RKKYKDRRK-NH_2	0.61	>200	>328
3	RKKYKERRK-NH_2	0.87	>200	>230
4	RKKYKFRRK-NH_2	0.15	>200	>1333
5	RKKYKGRRK-NH_2	0.31	>200	>645
6	RKKYKHRRK-NH_2	0.29	16.2	56
7	RKKYKIRRK-NH_2	0.16	57.4	359
8	RKKYKKRRK-NH_2	0.22	>200	>909
9	RKKYKLRRK-NH_2	0.32	132.0	413
10	RKKYKMRRK-NH_2	0.16	>200	>1250
11	RKKYKNRRK-NH_2	0.29	>200	>690
12	RKKYKPRRK-NH_2	0.12	>200	>1667
13	RKKYKQRRK-NH_2	0.30	>200	>667
14	RKKYKSRRK-NH_2	0.15	>200	>1333
15	RKKYKTRRK-NH_2	0.73	>200	>274
16	RKKYKVRRK-NH_2	0.36	>200	>556
17	RKKYKWRRK-NH_2	0.20	26.7	134
18	RKKYKYRRK-NH_2	0.05	>200	>4000

[a] IC_{50} values were calculated from the linear part of inhibition curves and represent the mean obtained from at least two different experiments.

2). The highest selectivity (>4,000-fold) for MLCK inhibition compared to CaMPKII inhibition is found with peptide **18**. None of the peptides inhibited PKA. Thus, chemically diverse amino acid side chains at position 6 allow for relatively high affinity and selective inhibitory activity. While there was a slight bias in the chemical characteristics of the side chain at position 5, this is less evident at position 6 and is suggestive of conformational constraint as a potential factor in selectivity and affinity. However, it is not known from the screening data alone if the recursive library approach maintained the bias toward inhibition competitive with the peptide substrate.

Mechanism of Peptide Inhibition. The experimental conditions used in our screening assays were biased toward the detection of inhibitors that compete with the peptide substrate because we used a peptide substrate concentration close to the K_m value and an ATP substrate concentration approximately 10-fold above the estimated K_m value. To check if our reiterative library synthesis and screen resulted in an inhibitor that is competitive with peptide substrate, we determined the mode of MLCK inhibition by Arg-Lys-Lys-Tyr-Lys-Tyr-Arg-Arg-Lys-NH_2 (peptide **18**) by kinetic analysis. As shown (Figure 3A) in the double-reciprocal (Lineweaver−Burk) plots at different inhibitor concentrations, the lines intersect at the *y* axis, indicating that the inhibition is competitive with respect to the peptide substrate. A K_i of 52 ± 1 nM was calculated based on two independent experiments (Figure 3B). In control experiments, MLCK was assayed at several concentrations of peptide **18** and ATP (28.6−200 μM) using a fixed concentration of the peptide substrate (100 μM). Double-reciprocal plots were indicative of mixed noncompetitive/uncompetitive inhibition with respect to ATP (not shown). Finally, inhibition of MLCK by peptide **18** was also found to be independent of CaM concentration (0.1−10 μM). All of these data indicate that peptide **18** has a selective effect on peptide substrate utilization by MLCK, does not interfere with kinase activation by CaM, does not have significant inhibitory activity with the closely related CaMPKII, and does not inhibit PKA. Thus, peptide **18** and related peptides represent a novel class of protein kinase inhibitors.

Structurally Constrained Peptide Inhibitors. Given the mode of inhibition and the structures that result in high selectivity toward MLCK, we can derive some insight into the features of the inhibitor that are key for selective kinase inhibition. For example, there is a clear preference for tyrosine at R4. However, the nature of the side chains at R5 and R6 that result in high selectivity toward inhibition of MLCK versus

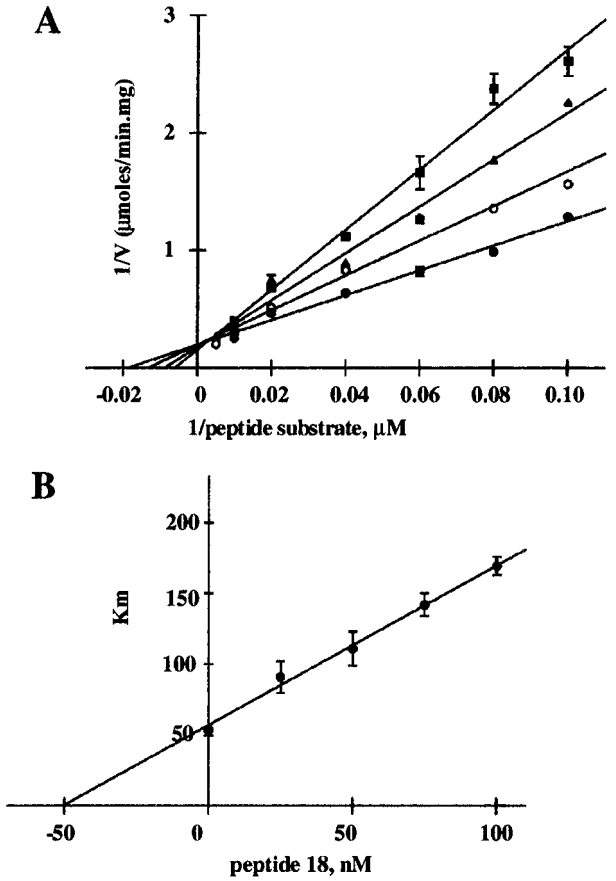


Figure 3. Kinetic analysis of inhibition of MLCK substrate phosphorylation by inhibitor peptide **18**. A. Double-reciprocal plots of the rate of substrate phosphorylation as a function of substrate concentration. Incorporation of ^{32}P into peptide substrate in the presence or absence of peptide **18** was measured as described under the Experimental Section. Each point is the mean of triplicate points in one representative experiment. Error bars indicate the standard errors and are not visible in the graph if the error is smaller than the symbol. The inhibitor peptide concentrations are 0 nM (●), 25 nM (○), 50 nM (▲), and 100 nM (■). B. Secondary plot of apparent K_m as a function of inhibitor concentration. The K_m values (mean ± SE) were estimated from linear regression of the double-reciprocal plots (panel A).

CaMPKII inhibition is diverse. Lysine and valine are preferred at R5 in the context of a pool that has tyrosine at R4 and any of the 18 amino acids at R6 (Figure 2). Similarly, side chains of tyrosine, phenylalanine, proline, methionine, and serine are preferred at R6 when R4 and R5 are restricted to tyrosine and lysine (Table 2). The poorer activity of glycine in this context indicates that extensive flexibility at these positions is probably not desirable. Although there is not a simple correlation with the chemical properties (charge, length) of the side chain present at these positions, there is the possibility of an interdependence of R5 and R6 (a nearest neighbor effect) and there is an apparent need for some conformational restriction, as suggested by the need for amino acids with side chains larger than glycine. We hypothesized, therefore, that residues 5 and 6 might contribute conformational constraints that restrict interactions with the different kinases, resulting in selectivity. While a three-dimensional structure is not available for MLCK, structures of two other Ser/Thr protein kinases with peptides bound in the active site are available.[24] The most relevant model is that of the catalytic subunit of phosphorylase kinase because it is a CaM-regulated protein kinase. In the phosphorylase kinase structure, the peptide substrate (Arg-Gln-Met-Ser-Phe-Arg-Leu) is in an extended conformation.[24b] Our screening results with changes at residue 5 or 6 are consistent with a requirement for such an extended conformation of the MLCK inhibitors. As a test of this possibility, we sought to incorporate a constrained residue that might allow a better interpretation of the results and potentially provide a starting point for future attempts at peptidomimetics. While there are many nonstandard amino acids available, one that has been used to mimic an extended conformation and to give an increase in selectivity is 4-aminocyclohexanecarboxylic acid.[25] Analysis of possible conformations of the commercially available *cis*-4-aminocyclohexanecarboxylic acid using the Insight/Discover molecular modeling suite revealed that the insertion of *cis*-4-aminocyclohexanecarboxylic acid can potentially make a backbone angle of 120−135°, resulting in a slightly bent conformation. The calculated nitrogen−carbonyl carbon distance of a *cis*-4-aminocyclohexanecarboxylic acid model is 5−5.1 Å, compared to 2.5−3 Å for an α-amino acid. Therefore, the use of *cis*-4-aminocyclohexanecarboxylic acid potentially offers novel angle and distance constraints in the backbone of a peptide and has been used successfully in attempts to improve peptide ligand selectivity.

To explore the effect of *cis*-4-aminocyclohexanecarboxylic acid at different positions in the backbone of peptide **18** on its kinase inhibitory activity, peptides containing *cis*-4-aminocyclohexanecarboxylic acid at positions 4, 5, and 6 and multiple combinations at these positions were synthesized and tested for inhibition of MLCK and CaMPKII. Peptide **19**, with *cis*-4-aminocyclohexanecarboxylic acid in position 5, was as active and selective as peptide **18** (Table 3). Peptide **22**, with two *cis*-4-aminocyclohexanecarboxylic acid residues at positions 5 and 6, has an IC_{50} for MLCK only 2-fold different from peptides **18** and **19**. Consistent with the screening of the combinatorial libraries, peptides **20**, **23**, **24**, and **25** (with *cis*-4-aminocyclohexanecarboxylic acid at position 4) are less effective MLCK inhibitors (Table 3) than peptides **19**, **21**, and **22** (with tyrosine at position 4). All peptides containing *cis*-4-aminocyclohexanecarboxylic acid are poor CaMPKII inhibitors, with $IC_{50} > 200$ μM. Thus, the selectivity factors (Table 3) for MLCK versus CaMPKII inhibition by these peptides are lower estimates.

Discussion

The discovery of novel peptide inhibitors that have better selectivity and inhibitory activity for MLCK versus CaMPKII than currently available peptide or nonpeptide inhibitors provides a proof-of-principle for a multidisciplinary approach that combines mechanistic knowledge from functional genomics with recursive combinatorial chemistry technologies and functional diversity in activity screens. Key considerations for success were the choice of an appropriate starting sequence based on the functional genomics analysis and the use of multiple activity analyses for each library as a basis for the design of the focused libraries. The recursive screening of focused combinatorial libraries

Table 3. Specificity of MLCK Inhibition by Selected Peptides

peptide	sequence	IC_{50}, μM[a]		
		MLCK	CaMPKII	ratio IC_{50}(CaMPKII)/IC_{50}(MLCK)
19	RKKY(Ach)YRRK-NH_2[b]	0.04	>200	>5000
20	RKK(Ach)KYRRK-NH_2	1.04	>200	>192
21	RKKYK(Ach)RRK-NH_2	0.27	>200	>741
22	RKKY(Ach)(Ach)RRK-NH_2	0.11	>200	>1818
23	RKK(Ach)(Ach)YRRK-NH_2	2.97	>200	>67
24	RKK(Ach)K(Ach)RRK-NH_2	20.0	>200	>10
25	RKK(Ach)(Ach)(Ach)RRK-NH_2	20.0	>200	>10

[a] IC_{50} values were calculated from the linear part of inhibition curves and represent the mean obtained from at least two different experiments. [b] (Ach), *cis*-4-aminocyclohexanecarboxylic acid.

based on the lead peptide sequence resulted in an enhancement of more than 20-fold in the IC_{50} for MLCK and greater than a 50-fold increase in selectivity for inhibition of MLCK over CaMPKII. The genome perspective, which is being provided by the rapidly accumulating body of data on potential open reading frames (ORFs) and changes in gene expression in response to various stimuli, provides a starting point and a vast resource for the potential extension of such a multidisciplinary approach to other enzymes that have catalytic and autoinhibitory domains in the same ORF. The combined application of functional genomics, combinatorial peptide chemistry, and high-throughput differential activity screens and enzyme kinetics needs to be tried with other enzymes in order to demonstrate the broader utility of the approach. However, the success with the CaM-regulated protein kinase family, which has not yielded to some of the approaches used for other protein kinases, provides a proof-of-principle for its potential utility with signal transduction pathways with established roles in organismal homeostasis.

The peptide inhibitors can be used with other protein kinase inhibitors and enzymology measurements to dissect the contribution of various signal transduction pathways to biological responses. For example, the response of cells and tissues to agonist or antagonist treatment is analyzed by rapid cell harvest at defined times after treatment, followed by immediate quantitative analysis of changes in a diverse array of enzyme activities. Because of the crude nature of the extract and the technical demand for use of peptide substrates to measure kinase activity, there is an inherent variability among repeat biological experiments. This variability is partially due to phosphorylation of endogenous substrates during the course of the enzyme reaction and overlapping peptide substrate recognition properties of the kinases, especially among a subgroup of CaM-regulated kinases. The ability to run control reactions which include addition of selective inhibitors is invaluable in the deconvolution of the data from such complex biological analyses. Newer technologies[26] allow the introduction of such peptide inhibitors into cultured cells for the inhibition of kinase activity,[23] providing additional potential uses.

In addition to the immediate uses of the peptide inhibitors, the profile of context-dependent activity for each amino acid position in the core tripeptide, including the *cis*-4-aminocyclohexanecarboxylic acid derivatives, provides a firm foundation for current studies of peptidomimetic analogues that retain inhibitory selectivity. For example, the activity screen conditions used in this study were chosen to bias competition with peptide substrate, versus nucleotide substrate as used in many other investigations. Kinetic analyses of peptide **18** demonstrated that it is competitive with peptide substrate. Therefore, a logical possibility for future investigations is to combine targeting to both substrate sites simultaneously in attempts to develop novel classes of inhibitors. Although peptide **18** was chosen for further analysis, it was only one of a series of peptides found during the screen that are more selective than currently available inhibitors of MLCK. On the basis of insight into the relation of inhibitor activity with structure from the recursive library screens, the unusual amino acid *cis*-4-aminocyclohexanecarboxylic acid was used in specific positions of the sequence with retention of both selectivity and inhibitory activity. The constraints inherent in *cis*-4-aminocyclohexanecarboxylic acid and its potential ability to promote novel extended conformations in peptides suggest that it provides a peptide backbone and orientation of functional groups that are favorable for selective inhibition of MLCK versus other protein kinases. The three-dimensional structure of MLCK is not available, thus precluding the efficient use of structure-assisted inhibitor development in conjunction with the combinatorial chemistry, and three-dimensional homology models of MLCK based on distantly related serine/threonine protein kinases did not offer sufficient information to fully explain the structure−activity correlates. Nonetheless, precedents with other systems[27] suggest that *cis*-4-aminocyclohexanecarboxylic acid and other conformationally constrained derivatives might provide new scaffolds for further development of peptidomimetic inhibitors.

Conclusion

A generalizable multistep approach for the development of potent and selective inhibitors of proteins which regulate their activity by means of autoinhibitory sequences was tested with a prototype CaM-regulated enzyme. On the basis of the identification of the appropriate lead sequence by functional genomic analyses, the activity and selectivity of peptide inhibitors can be enhanced using focused recursive combinatorial peptide library screening with multiple enzymes. The insight into structure−activity data derived from the screen can be used as the basis for substitution of unusual amino acids at selected positions with retention of affinity and selectivity. The inhibitors generated by this proof-of-principle study add to the available tools for the dissection of the linkage between protein kinases and cell function.

Experimental Section

Combinatorial Peptide Resin Mix and Split Protocol. The solid-phase mix and split technology[28] was used to generate peptide mixtures such that each resin bead has a single peptide. For 1.3 g of resin, the collection vessel on the Advanced ChemTech 357 synthesizer was filled with 80 mL of dimethylformamide:dichloromethane (1:1, v/v) and mixed for 3 min. The resin sampling syringe then dispensed 3.5 mL of the suspension to each of 18 reaction vessels. The collection vessel was drained and then filled again with 70 mL of dimethylformamide:dichloromethane (1:1, v/v) and 3.5 mL of suspension transferred to each reaction vessel and then drained. This second transfer was repeated two additional times. For recombination of the 18 pools, 3 mL of dimethylformamide:dichloromethane (1:1, v/v) was dispensed to each reaction vessel and mixed for 1 min. Using the resin sampling syringe 3 mL of suspension was transferred from each reaction vessel to the collection vessel. The dispense and transfer process was repeated two additional times.

General Repetitive Solid-Phase Synthesis Protocol. Peptides and peptide libraries were synthesized by solid-phase peptide synthesis techniques using Fmoc chemistry. Fmoc amino acids were from Advanced ChemTech. The side chains of His, Asn, and Gln were protected with trityl; the guanyl group on Arg was protected with 2,2,5,7,8,-pentamethylchroman-6-sulfonyl; Ser, Thr, Asp, and Glu were protected with *tert*-butyl; Lys and Trp side chains were blocked with *tert*-butyloxycarbonyl.

The N-α-Fmoc group was cleaved at each step with 25% (v/v) piperidine:dimethylformamide for 3 min with continuous mixing followed by another treatment with fresh reagent for 12 min with continuous mixing. The resin was washed with dimethylformamide (3×), methanol (2×), and dimethylformamide (3×). Fmoc amino acids were dissolved in *N*-methylpyrrolidone (NMP) at 0.5 M containing 0.5 M hydroxybenzotriazole (HOBt). A 6-fold molar excess of amino acid was used at each coupling step. The first coupling reaction was done by adding an amount of diisopropylcarbodiimide (DIC) in NMP equivalent to the amount of Fmoc amino acid and mixing for 45 min. The second coupling step was done by adding an equivalent of *O*-benzotriazole-*N*,*N*,*N*′,*N*′−tetramethyluroniumhexafluorophosphate (HBTU) and 2 equiv of diisopropylethylamine in NMP and mixing for 30 min. After the coupling steps the peptide-resin was washed three times with dimethylformamide.

Combinatorial Library 1 Synthesis, Cleavage, and Characterization. Rink resin, 1.3 g (Advanced ChemTech; 0.56 mmol of N/g), containing the carboxyl-terminal tripeptide Arg-Arg-Lys was prepared in a single batch using Fmoc-protected amino acids (Advanced ChemTech). A 3-fold excess of amino acid was used in each of the two coupling steps for the tripeptide batch synthesis, while a 6-fold excess was used for each of the 18 pools in the subsequent cycles of synthesis. The tripeptide resin was mixed and split into 18 vessels, and the next amino acid was coupled (one per vessel). Using the same mix and split protocol, synthesis was done at position 5 of the peptide. After another mix and split round, one of 18 amino acids was coupled to the resin in each vessel for position 4. Each of the 18 vessels then had Lys, Lys, and Arg added in three synthetic rounds. The final weight of resin containing each pool was in the range of 94−184 mg. The peptides were cleaved from the resin for 3.5 h in 0.8 mL of trifluoroacetic acid (TFA):anisole:dithioethanol (90:5:5, v/v/v) at room temperature. Peptides were precipitated with ethyl ether, washed, and then extracted with 20% (v/v) acetic acid and freeze-dried. Crude peptide mixtures of each pool were dissolved in 1 mL of water and quantitated by amino acid analysis normalized to Arg after acid hydrolysis. The yield of peptide material in the pools was in the range of 45.5−81.3% of theoretical.

Combinatorial Library 2 Synthesis, Cleavage, and Characterization. As with the first combinatorial library, 1.3 g of Rink resin (Advanced ChemTech; 0.52 mmol of N/g), containing the carboxyl-terminal tripeptide Arg-Arg-Lys was prepared. Resin was divided into 18 vessels. One round of split and mix technology was used for random synthesis at position 6. For position 5, one of 18 amino acids was coupled to the resin in each vessel, and the remaining Tyr, Lys, Lys, and Arg were coupled to each of the 18 pools as above. The final weight of each resin pool was 115−215 mg; 70 mg of each resin was cleaved for 3.5 h in 0.8 mL of the mixture TFA:anisole:dithioethanol (90:5:5, v/v/v) at room temperature. Peptides were precipitated with ethyl ether, washed, extracted, and dried as described above. The crude peptides were dissolved in 1 mL of water and quantitated by amino acid analysis after acid hydrolysis normalized to Arg and Tyr. The yield of peptides was in the range of 44.4−64.3% of theoretical.

Solid-Phase Synthesis of Individual Peptides. In the parallel syntheses, 50 mg of Rink resin (Advanced ChemTech; 0.52 mmol of N/g) containing the carboxyl-terminal Arg-Arg-Lys-NH_2 was used for each of 18 vessels. Amino acid synthesis was done for 6 additional cycles so that position 6 contained one of 18 amino acids and followed by Lys, Tyr, Lys, Lys, and Arg. Peptides were cleaved and processed as described above. The yield of peptide pools was in the range of 24−50% of theoretical.

Peptides were further purified by HPLC chromatography on a preparative Chromosorb C18 column using gradients of 0.1% (v/v) TFA in water and 60% (v/v) aqueous acetonitrile containing 0.08% (v/v) TFA. Fractions of the desired peptide were freeze-dried, dissolved in water, and quantitated by amino acid analysis after acid hydrolysis.[12] Peptides were characterized by analytical HPLC on a Chromosorb C8 column using aqueous acetonitrile gradients. Purified peptides were also characterized by matrix-assisted laser-desorption mass spectrometry (MALDI-TOF) (Table 1). Peptide purity was at least 88−95%.

Fmoc-*cis*-4-aminocyclohexanecarboxylic acid was prepared in 85% yield from Fmoc-OSu (Aldrich) and *cis*-4-aminocyclohexanecarboxylic acid (Aldrich) as described.[25] The derivatized amino acid was an oil, appeared to be >90% homogeneous by TLC in chloroform:methanol:acetic acid (89:10:1, v/v/v) and was used without further purification.

Library Validation. We performed multiple amino acid analyses of the combinatorial library pools hydrolyzed for 24 or 72 h to quantitate the amount of peptides present. This was based upon normalization to the fixed amino acids in the peptide (e.g., arginine and/or tyrosine). The normalization also indicated that the pools contained a relatively unbiased representation of all of the peptides. If the constituent peptides of the pool were not evenly represented, the activity of the pool might have been better or worse than we observed. To test this possibility, we created a mock pool equivalent to pool **2h** using an equimolar mix of the individually purified peptides in Table 1 and tested it for MLCK inhibition. This mixture exhibited an IC_{50} of 0.11 μM, comparable to that of pool **2h** (IC_{50} = 0.09 μM). Therefore, pool **2h** appears to be an unbiased representation of the peptide constituents.

Enzyme Inhibition Assays. MLCK was purified from chicken gizzard tissue,[13] recombinant CaMPKII was from New England Biolabs, and the catalytic subunit of PKA was from Promega. CaM was made as previously described.[13] All assays were done in a final volume of 50 μL. Each point was tested in duplicate. The standard assay conditions were 50 mM HEPES, 0.2 mM [γ-^{32}P]ATP (specific activity 200−300 cpm/pmol), 1 mM DTT, 1 mg/mL bovine serum albumin, 5 mM $MgCl_2$, 150 mM KCl, 15 mM NaCl, pH 7.5, with or without inhibitors. Synthetic substrate concentrations, KKRPQRATSN-VFAM-NH_2 for MLCK, PLRRTLSVAA-NH_2 for CaMPKII, and LRRASLG for PKA, were 20 μM. The reactions were initiated with the addition of 5 μL of enzyme (PKA) or enzyme:CaM complex (for MLCK and CaMPKII). Enzyme:CaM complexes were preformed by incubation of enzyme in ice for 1 min in the presence of 1 μM CaM and 5 μM $CaCl_2$ (for MLCK) or 30 μM CaM and 2 mM $CaCl_2$ (for CaMPKII). Assay conditions were 20 min, 25 °C for MLCK; 8 min, 25 °C for CaMPKII; 6 min, 25 °C for PKA. Aliquots of each reaction tube were spotted into phosphocellulose filter paper (P-81, Whatman). Filters were washed in 75 mM phosphoric acid solution, followed by

95% ethanol, dried, and placed into vials containing 10 mL of Ecoscint O (National Diagnostics). Radioactivity was determined by counting vials in a Beckman 6500 liquid scintillation counter. No quenching corrections were applied. Data were transformed as percent of the maximal enzyme activity, where enzyme activity in the absence of inhibitors was taken as 100%. IC_{50} values were calculated by a linear regression data analysis on data points corresponding to 15%−85% of maximal activity. This analysis was done using InPlot version 4.0 (GraphPad Software Inc., 1992).

Kinetic Analysis of MLCK Inhibition. MLCK inhibition by peptide **18** was assayed in the presence of 25−100 nM peptide. Substrate concentration was varied from 10 to 200 μM, and ATP concentration was fixed (200 μM). For analysis of ATP kinetics, MLCK inhibition by peptide **18** was assayed in the presence of 25−200 nM peptide, ATP concentration was varied from 28.6 to 200 μM, and peptide substrate concentration was fixed (100 μM). Assays were done as described above, except that the time of assay was 10 min at 25 °C. Data were analyzed by double-reciprocal (Lineweaver−Burk) plots using InPlot version 4.0 (GraphPad Software Inc., 1992).

Computational Analysis. Isomers of *cis*- and *trans*-4-aminocyclohexanecarboxylic acid were constructed using the fragment and group libraries in InsightII (Molecular Simulations, Inc). For a generic model, the amino-terminus was acetylated, and the carboxyl-terminus was *N*-methylamide. The initial models were structurally optimized with Discover (Molecular Simulations) using Newton−Rhapson followed by conjugate gradient (1000 cycles or till convergence) minimizer routines. Atom types, partial charges, and potentials were from the CVFF library[29] (Molecular Simulations). Torsional energies were evaluated using real-time monitoring within InsightII of total van der Waals and electrostatic electrostatics while changing a single torsional angle.

Acknowledgment. This work is supported in part by National Institutes of Health (NIH) Grant GM 30861. D.M.W. is the John G. Searle Professor of Molecular Biology and Biochemistry and a member of the Robert H. Lurie Comprehensive Cancer Center. The authors thank J. Schultz for technical assistance and Dr. L. J. Van Eldik for critical comments on the manuscript.

Supporting Information Available: Amino acid analysis data for pools **1a**−**1r** from the first combinatorial peptide library and pools **2a**−**2r** from the second combinatorial peptide library. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Bourlot, A.-S.; Sanchez, I.; Dureng, G.; Guillaumet, G.; Massingham, R.; Monteil, A.; Winslow, E.; Pujol, M. D.; Merour, J.-Y. New Substituted 1,4-Benozoxazine Derivatives with Potential Intracellular Calcium Activity. *J. Med. Chem.* **1998**, *41*, 3142−3158.

(2) (a) Oates, J. A. Antihypertensive Agents and the Drug Therapy of Hypertension. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*; Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., Gilman, A. G., Eds.; McGraw-Hill: New York, 1996; pp 780−808. (b) Robertson, R. M.; Robertson, D. Drugs Used for the Treatment of Myocardia Ischemia. *Ibid*; pp 759−779.

(3) Nelson, M. R.; Chazin, W. J. Calmodulin as a Calcium Sensor. In *Calmodulin and Signal Transduction*; Van Eldik, L. J., Watterson, D. M., Eds.; Academic Press: San Diego, 1998; pp 17−64.

(4) Lukas, T. J.; Mirzoeva, S.; Watterson, D. M. Calmodulin-Regulated Protein Kinases. In *Calmodulin and Signal Transduction*; Van Eldik, L. J., Watterson, D. M., Eds.; Academic Press: San Diego, 1998; pp 66−168.

(5) (a) Wilson, K.; McCaffrey, P. G.; Hsiao, K.; Pazhanisamy, S.; Galullo, V.; Bemis, G. W.; Fitzgibbon, M. J.; Caron, P. R.; Murcko, M. A.; Su, M. S. The Structural Basis for the Specificity of Pyridinylimidazole Inhibitors of p38 MAP Kinase. *Chem. Biol.* **1997**, *4*, 423−431. (b) Badger, A. M.; Bradbeer, J. N.; Votta, B.; Lee, J. C.; Adams, J. L.; Griswold, D. E. Pharmacological Profile of SB 203580, a Selective Inhibitor of Cytokine Suppressive Binding Protein/p38 Kinase, in Animal Models of Arthritis, Bone Resorption, Endotoxin Shock and Immune Function. *J. Pharmacol. Exp. Ther.* **1996**, *279*, 1453−1461.

(6) Lee, J. C.; Laydon, J. T.; McDonnell, P. C.; Galagher, T. F.; Kumar, S.; Green, D.; McNulty, D.; Blumenthal, M. J.; Heys, J. R.; Landvatter, S. W.; Strickler, J. E.; McLaughlin, M. M.; Siemens, I. R.; Fisher, S. M.; Livi, G. P.; White, J. R.; Adams, J. L.; Young, P. R. A Protein Kinase Involved in the Regulation of Inflammatory Cytokine Biosynthesis. *Nature* **1994**, *372*, 739−746.

(7) Casnellie, J. E. Protein kinase inhibitors: Probes for the Functions of Protein Phosphorylation. *Adv. Pharmacol.* **1991**, *22*, 167−203.

(8) Hashimoto, Y.; Nakayama, T.; Teramoto, T.; Kato, H.; Watanabe, T.; Kinoshita, M.; Tsukamoto, K.; Tokunaga, K.; Kurokawa, K.; Nakanishi, S.; Matsuda, Y.; Nonomura, Y. Potent and Preferential Inhibition of Ca^{2+}/Calmodulin- Dependent Protein Kinase II by K252a and its Derivative, KT5926. *Biochem. Biophys. Res. Commun.* **1991**, *181*, 423−429.

(9) Reig, J. A.; Viniegra, S.; Ballesta, J. J.; Palmero, M.; Gutierrz, L. M. Naphthalenesulfonamide Derivatives ML9 and W7 Inhibit Catecholamine Secretion in Intact and Permeabilized Chromaffin Cells. *Neurochem. Res.* **1993**, *18*, 317−323.

(10) Caufield, M. P.; Robbins, J.; Sim, J. A.; Brown, D. A.; MacNeil, S.; Blackburn, G. M. The Naphthalenesulphonamide Calmodulin Antagonist W7 and its 5-iodo-1-C_8 Analogue Inhibit Potassium and Calcium Currents in NG108-15 Neuroblastoma x Glioma Cells in a Manner Possibly Unrelated to Their Antagonism of Calmodulin. *Neurosci. Lett.* **1991**, *125*, 57−61.

(11) Li, G.; Hidaka, H.; Wollheim, C. B. Inhibition of Voltage-Gated Ca^{2+} Channels and Insulin Secretion in HIT Cells by the Ca^{2+}/Calmodulin-Dependent Protein Kinase II inhibitor KN-62: Comparison with Antagonists of Calmodulin and L-type Ca^{2+} Channels. *Mol. Pharmacol.* **1992**, *42*, 489−498.

(12) Lukas, T. J.; Burgess, W. H.; Prendergast, F. G.; Lau, W.; Watterson, D. M. Calmodulin Binding Domains: Characterization of a Phosphorylation and Calmodulin Binding Site from Myosin Light Chain Kinase. *Biochemistry* **1986**, *25*, 1458−1464.

(13) Shoemaker, M. O.; Lau, W.; Shattuck, R. L.; Kwiatkowski, A. P.; Matrisian, P. E.; Guerra-Santos, L.; Wilson, E.; Lukas, T. J.; Van Eldik, L. J.; Watterson, D. M. Use of DNA Sequence and Mutant Analyses and Antisense Oligodeoxynucleotides to Examine the Molecular Basis of Non-Muscle Myosin Light Chain Kinase Autoinhibition, Calmodulin Recognition, and Activity. *J. Cell Biol.* **1990**, *111*, 1107−1125.

(14) (a) Tanaka, M.; Ikebe, R.; Matsuura, M.; Ikebe, M. Pseudosubstrate Sequence May Not be Critical for Autoinhibition of Smooth Muscle Myosin Light Chain Kinase. *EMBO J.* **1995**, *14*, 2839−2846. (b) Krueger, J. K.; Padre, R. C.; Stull, J. T. Intrasteric Regulation of Myosin Light Chain Kinase. *J. Biol. Chem.* **1995**, *270*, 16848−16853.

(15) Goldberg, J.; Nairn, A. C.; Kuriyan, J. Structural Basis for the Autoinhibition of Calcium/Calmodulin-Dependent Protein Kinase I. *Cell* **1996**, *84*, 875−887.

(16) Lukas, T. J.; Haiech, J.; Lau, W.; Craig, T. A.; Zimmer, W. E.; Shattuck, R. L.; Shoemaker, M. O.; Watterson, D. M. Calmodulin and Calmodulin-Regulated Protein Kinases as Transducers of Intracellular Calcium Signals. *Cold Spring Harbor Symp. Quant. Biol.* **1988**, *53*, 185−193.

(17) (a) Kemp, B. E.; Pearson, R. B.; Guerriero, V.; Bagchi, I. C.; Means, A. R. The Calmodulin Binding Domain of Chicken Smooth Muscle Myosin Light Chain Kinase Contains a Pseudosubstrate Sequence. *J. Biol. Chem.* **1987**, *262*, 2542−2548. (b) Pearson, R. B.; Wettenhall, R. E. H.; Means, A. R.; Hartshorne, D. J.; Kemp, B. E. Autoregulation of Enzymes by Pseudosubstrate Prototypes: Myosin Light Chain Kinase. *Science* **1988**, *241*, 970−973.

(18) (a) Foster, C. J.; Johnston, S. A.; Sunday, B.; Gaeta, F. C. A. Potent Peptide Inhibitors of Smooth Muscle Myosin Light Chain Kinase: Mapping of the Pseudosubstrate and Calmodulin Binding Domains. *Arch. Biochem. Biophys.* **1990**, *280*, 397−404. (b) Ikebe, M.; Reardon, S.; Fay, F. S. Primary Structure Required for the Inhibition of Smooth Muscle Myosin Light Chain Kinase. *FEBS Lett.* **1992**, *312*, 245−248.

(19) Bagchi, I. C.; Kemp, B. E.; Means, A. R. Intrasteric Regulation of Myosin Light Chain Kinase: The Pseudosubstrate Prototope Binds to the Active Site. *Mol. Endocrinol.* **1992**, *6*, 621−626.

(20) Smith, M. K.; Colbran, R. J.; Soderling, T. R. Specificities of Autoinhibitory Domain Peptides for Four Protein Kinases. Implications for Intact Cell Studies of Protein Kinase Function. *J. Biol. Chem.* **1990**, *265*, 1837−1840.

(21) Houghten, R. A.; Pinilla, C.; Blondelle, S.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. Generation and Use of Synthetic Peptide Combinatorial Libraries for Basic Research and Drug Discovery. *Nature* **1991**, *354*, 84−86.

(22) King, D. S.; Fields, C. G.; Fields, G. B. A Cleavage Method Which Minimizes Side Reactions Following Fmoc Solid-Phase Peptide Synthesis. *Int. J. Pept. Protein Res.* **1990**, *36*, 255−266.
(23) Lukas, T. J.; Mirzoeva, S.; Watterson, D. M. Unpublished results.
(24) (a) Knighton, D. R.; Zheng, J.; Ten Eyck, L. F.; Xuong, N.-H.; Taylor, S. S.; Sowadski, J. M. Structure of a Peptide Inhibitor Bound to the Catalytic Subunit of Cyclic Adenosine Monophosphate-Dependent Protein Kinase. *Science* **1991**, *253*, 414−420. (b) Lowe, E. D.; Noble, M. E. M.; Skamnaki, V. T.; Oikonomakos, N. G.; Owen, D. J.; Johnson, L. N. The Crystal Structure of a Phosphorylase Kinase Peptide Substrate Complex: Kinase Substrate Recognition. *EMBO J.* **1997**, *16*, 6646−6658.
(25) Snyder, K. R.; Murray, T. F.; DeLander, G. E.; Aldrich, J. V. Synthesis and Opioid Activity of Dynorphin A-(1−13)NH_2 Analogues Containing *cis*- and *trans*-4-Aminocyclohexane Carboxylic Acid. *J. Med. Chem.* **1993**, *36*, 1100−1103.
(26) (a) Derossi, D.; Calvet, S.; Trembleau, A.; Brunissen, A.; Chassaing, G.; Prochiantz, A. Cell Internalization of the Third Helix of the Antennapedia Homeodomain is Receptor Independent. *J. Biol. Chem.* **1996**, *271*, 18188−18193. (b) Theodore, L.; Derossi, D.; Chassaing, G.; Llirbat, B.; Kubes, M.; Jordan, P.; Chneiweiss, H.; Godement, P.; Prochiantz, A. Intraneuronal Delivery of Protein Kinase C Pseudosubstrate Leads to Growth Cone Collapse. *J. Neurosci.* **1995**, *15*, 7158−7167.
(27) (a) Kick, E. K.; Roe, D. C.; Skillman, A. G.; Liu, G.; Ewing, T. J. A.; Sun, Y.; Kuntz, I. D.; Ellman, J. A. Structure-Based Design and Combinatorial Chemistry Yield Low Nanomolar Inhibitors of Cathepsin D. *Chem. Biol.* **1997**, *4*, 297−307. (b) Feng, S.; Kapoor, T. M.; Shirai, F.; Combs, A. P.; Schreiber, S. L. Molecular Basis for the Binding of SH3 Ligands with Non-peptide Elements Identified by Combinatorial Synthesis. *Chem. Biol.* **1996**, *3*, 661−670. (c) Alfaro-Lopez, J.; Yuan, W.; Phan, B. C.; Kamath, J.; Lou, Q.; Lam, K. S.; Hruby, V. J. Discovery of a Novel Series of Potent and Selective Substrate-Based Inhibitors of $p60^{c-src}$ Protein Tyrosine Kinase: Conformational and Topographical Constraints in Peptide Design. *J. Med. Chem.* **1998**, *41*, 2252−2260.
(28) (a) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. A New Type of Synthetic Peptide Library for Identifying Ligand-Binding Activity. *Nature* **1991**, *354*, 82−84. (b) Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. General Method for Rapid Synthesis of Multicomponent Peptide Mixtures. *Int. J. Pept. Protein Res.* **1991**, *37*, 487−493.
(29) (a) Dauber-Osguthorpe, P.; Roberts, V. A.; Osguthorpe, D. J.; Wolff, J.; Genest, M.; Hagler, A. T. Structure and Energetics of Ligand Binding to Proteins: E. coli Dihydrofolate Reductase-Trimethoprim, a Drug-Receptor System. *Proteins* **1988**, *4*, 31−47. (b) Hagler, A. T.; Stern, P. S.; Sharon, R.; Becker, J. M.; Naider, F. Computer Simulation of the Conformational Properties of Oligopeptides. Comparison of Theoretical Methods and Analysis of Experimental Results. *J. Am. Chem. Soc.* **1979**, *101*, 6842−6852.

JM980573A