ARTICLE

ACS chemical biology

# Use of Docking Peptides to Design Modular Substrates with High Efficiency for Mitogen-Activated Protein Kinase Extracellular Signal-Regulated Kinase

**Neychelle Fernandes[†], Denise E. Bailey[‡], David L. VanVranken[‡], and Nancy. L. Allbritton[†,‡,§,*]**

[†]Department of Chemical Engineering and Materials Science and [‡]Department of Chemistry, University of California, Irvine, California 92697, and [§]Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina 27599

**ABSTRACT** The mitogen-activated protein kinase extracellular regulated kinase (ERK) plays a key role in the regulation of cellular proliferation. Mutations in the ERK cascade occur in 30% of malignant tumors. Thus understanding how the kinase identifies its cognate substrates as well as monitoring the activity of ERK is central to cancer research and therapeutic development. ERK binds to its protein targets, both downstream substrates and upstream activators, *via* a binding site distinct from the catalytic site of ERK. The substrate sequences that bind, or dock, to these sites on ERK influence the efficiency of phosphorylation. For this reason, simple peptide substrates containing only phosphorylation sequences typically possess low efficiencies for ERK. Appending short docking peptides derived from full-length protein substrates and activators of ERK to a phosphorylation sequence increased the affinity of ERK for the phosphorylation sequence by as much as 200-fold while only slightly diminishing the maximal velocity of the reaction. The efficiency of the phosphorylation reaction was increased by up to 150-fold, while the specificity of the substrate for ERK was preserved. Simple modular peptide substrates, which can be easily tailored to possess high phosphorylation efficiencies, will enhance our understanding of the regulation of ERK and provide a tool for the development of new kinase assays.

Mitogen-activated protein (MAP) kinases, which include the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinases, are key regulators of cell proliferation and stress survival (*1, 2*). Increases in MAP kinase activity have been linked to proliferative disorders including cancer (*3–6*). In particular, activation of ERK is implicated in breast, ovarian, and prostate cancers (*7*). The activation of ERK is initiated by a cascade of sequentially activated proteins. The Ras G-proteins (H-, K-, and N-Ras) phosphorylate and activate Raf kinases (A-, B-, and C-Raf), which in turn phosphorylate the MAP kinase kinases 1 and 2 (MEK1 and -2). MEK1 and -2 phosphorylate and activate the MAP kinase ERK (*2, 8*). Activated ERK phosphorylates a number of substrates including ribosomal protein S6 kinases (RSKs) and transcription factors, for example, the ETS family of proteins that control and regulate cell growth and differentiation (*9–13*). As with other MAP kinases, ERK is a compact kinase without additional regulatory domains. The activity of ERK is tightly controlled by the phosphorylation of both a tyrosine and a threonine residue in the activation loop (*14–16*). With multiple MAP kinases present in the same cellular space, mechanisms must exist to prevent the different MAP kinase pathways from inappropriately interacting with each other. Many of the factors controlling the specificity and efficiency of ERK are not fully understood. Analysis of protein and peptide substrates demonstrate that ERK is a proline-directed serine or threonine kinase with a phosphorylation consensus motif of Ser/Thr-Pro (*12, 17*). However, this motif alone is insufficient to explain the efficiency

*Corresponding author, nlallbri@unc.edu.

Received for review July 28, 2007 and accepted September 19, 2007.

Published online October 6, 2007

10.1021/cb700158q CCC: $37.00

and specificity of ERK for its substrates, since other MAP kinases (p38 kinase and JNK), as well as cyclin-dependent kinases (CDKs), also phosphorylate a similar consensus motif (*17*).

The specificity and efficiency of MAP kinases for their substrates is thought to be determined by high-affinity binding motifs on the protein substrate that are distinct from the consensus phosphorylation sequence (*18–31*). One of the earliest identified binding or docking motifs was the delta domain on the transcription factor c-Jun, which mediates the high-affinity binding of the MAP kinase JNK to c-Jun (*32, 33*). Various mutagenic and crystallographic studies have now identified discrete sequences of amino acids or docking motifs on many of the substrates of the MAP kinases. These docking motifs on the substrates are responsible for the specific interactions of the substrate with its appropriate MAP kinase (*19–31*). In addition, docking motifs on the substrates of ERK increase the efficiency of phosphorylation of the substrate (*18, 21–24, 28–31, 34*). The major docking motif on ERK substrates is the D domain, which possesses a consensus sequence $(Arg/Lys)_{2-3}$-$(X)_{1-6}$-$\Phi_A$-X-$\Phi_B$; where $\Phi_A$ and $\Phi_B$ are hydrophobic residues such as leucine, isoleucine, or valine and X is any amino acid (*18, 21, 22, 24, 27, 29*). The D motifs in ERK-binding proteins (substrates, MAP kinase kinases, and ERK phosphatases) bind to a complementary docking site on ERK composed of a highly acidic patch and a hydrophobic groove located on the opposite face of the kinase relative to the catalytic site (*18, 22, 24, 27, 29*). Docking motifs have been identified in substrates of p38 and JNK that are similar to but distinct from the docking domain on substrates of ERK. Mutating residues in the docking region of ERK to those found in p38 kinase or JNK changes the specificity of ERK to that of p38 or JNK, respectively. Mutation of specific residues on ERK, for example, two threonine residues in the acidic patch binding to the substrate D domain, enables ERK to phosphorylate substrates of the MAP kinase p38$\alpha$ and reduces the efficiency of phosphorylation of ERK substrates (*24*).

Biochemical and structural studies of docking interactions between MAP kinases and their substrates are key to developing a clear understanding of how MAP kinases recognize and efficiently phosphorylate appropriate substrates while rejecting inappropriate substrates. A goal of this work was to demonstrate a simple model system that yielded insights into the role of the docking interactions between ERK and its binding partners. Docking peptides derived from the D domain of downstream substrates or upstream kinase activators were linked to a peptide possessing the consensus phosphorylation sequence, and the contribution of each component to the binding affinity and the rate of phosphate transfer was assessed. Since short peptides with the phosphorylation consensus sequence alone possess low affinity ($K_M > 100$ $\mu$M) for ERK compared with full length substrate proteins, current peptide substrates are limited largely to assays of purified enzyme for which the enzyme concentration can be controlled and no competing enzymes are present. Thus a second goal was to determine whether the designed docked peptides could serve as high-efficiency peptide substrates for ERK (*35, 36*).

Development of efficient peptide substrates for ERK and other MAP kinases will yield new opportunities in the development of kinase assays. These substrates would broaden the range of peptide-based *in vitro* assays to utilize impure enzyme (cell lysates and tissue homogenates) and serve as a basis for the development of new kinase indicators to monitor ERK activity in intact cells. A wider range of FRET-based kinase indicators than that currently available would be enabled by the development of simple yet efficient peptide substrates for kinases that rely on docking interactions. For example, the FRET-based indicator strategy pioneered by Tsien and others (*37*) incorporates a peptide substrate sequence between two fluorescent proteins and adjacent to a phosphopeptide-binding motif. Others methods use phosphorylation of a peptide-based substrate to assist in reassembly of a circularly permutated fluorescent protein. These methods have been very successful for kinases such as protein kinase C and A, which derive much of their specificity from the residues surrounding the phosphorylation residue; however, the strategies are difficult to implement for kinases that derive the majority of their efficiency and specificity from docking sites distant from the phosphorylated residue (*38*). Efficient peptide substrates for ERK and other similar kinases would open the door to the development of new FRET-based indicators of this type. A second class of kinase indicators that rely on efficient peptide-based substrates are those developed by Lawrence and colleagues (*39*). These probes have high value in measurements on primary cells since they are not genetically encoded. Similarly, short peptide substrates have been

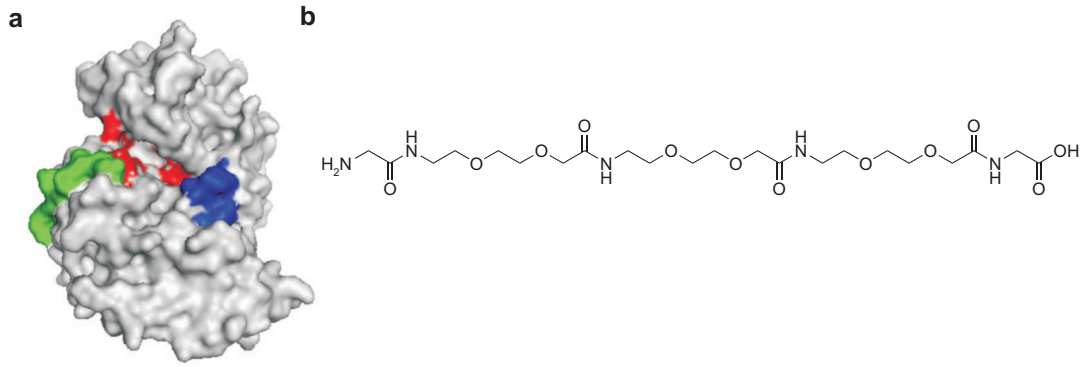


**Figure 1. A linker is required to connect the docking peptide to the substrate sequence. a) Structure of ERK2 (PDB 2FYS) indicating the residues interacting with the substrate D domain (green), substrate sequence (blue), and ATP (red). The image was created with Pymol (DeLano Scientific LLC). b) Shown is the linker $AOO_3$ with a glycine at both termini (Gly-$(AOO)_3$-Gly).**

used by Allbritton and colleagues (*40*) to report intracellular kinase activity, and this method would also benefit from the development of efficient peptide-based substrates for use as reporters of cellular enzyme activity.

## RESULTS AND DISCUSSION

**Components of the Designed ERK Substrates.** A series of peptide substrates consisting of four modules was designed. The first module was a fluorophore, fluorescein (5-FAM), used for detection and quantitation of the peptides. The second component was a peptide matching the ERK consensus phosphorylation motif and possessing a serine as the phosphoacceptor. The third piece was a peptide composed of the D domain docking sequence from either upstream activators of ERK (MAP kinase kinases) or downstream substrates of ERK. The final module was a linker bridging the docking peptide and the substrate peptide. The phosphorylation sequence selected was "TGPLSPGPF". This peptide was predicted to be the optimal peptide sequence for ERK by Songyang *et al.* (*17*) using an oriented, degenerate, peptide library. The sequence "TGPLSPGPF" possesses the minimum consensus motif Ser/Thr-Pro found in ERK substrates such as tyrosine hydroxylase and epidermal growth factor (*12, 17*). In addition, it also contains proline at the −2 position, which enhances the $V_{max}$ of peptide phosphorylation by ERK (*41*). The affinity ($K_M$) and the catalytic constant ($k_{cat}$) of a similar peptide "ATGPLSPGPFGRR" for ERK2 are 450 ± 230 μM and 120 ± 8 $min^{-1}$, respectively (*35*).

**Selection of the Docking Peptides.** The docking peptides were derived from the D domains of proteins with a range of binding affinities for ERK. Since the D domains of upstream activators of ERK play a major role in their binding to ERK, peptides derived from these domains may also exhibit enhanced binding to ERK (*21, 28, 42*). A docking peptide derived from the N-terminal 13 amino acids of the MAP kinase kinase MEK1 ($^{1}$MPKKKPTPIQLNP$^{13}$) was chosen since MEK1 binds ERK2 with an affinity of 29 μM (*28*). A docking peptide derived from the N-terminal 16 amino acids of MEK2 ($^{1}$MLARRKPVLPALTINP$^{16}$) was utilized as a potential intermediate-affinity peptide since MEK2 possesses an affinity of 9 μM for ERK2 (*28*). STE7, a MAP kinase kinase from the yeast *Saccharomyces cerevisiae*, interacts with mammalian ERK1 and ERK2 with a $K_D$ of 1 μM. Thus a peptide comprised of the N-terminal 18 amino acids ($^{2}$FQRKTLQRRNLKGLNLNL$^{19}$) was employed as a potential higher-affinity binding peptide than the peptide derived from MEK2 (*28*). A fourth docking peptide, $^{310}$PQKGRKPRDLELPL$^{323}$ was derived from the N-terminal 14 amino acids of the transcription factor ELK1, a substrate of ERK. The affinity for ERK of the ELK1 protein without the FXFP motif is 5 μM (*21*).

**Selection of the Linker Composition.** A linker was used to bridge the docking peptide to the substrate peptide since the D domain binding site and catalytic cleft on ERK are not immediately adjacent to one another (Figure 1, panel a). The linker also permitted the docking peptide and substrate to have flexibility in their relative orientations. Choosing the appropriate type and

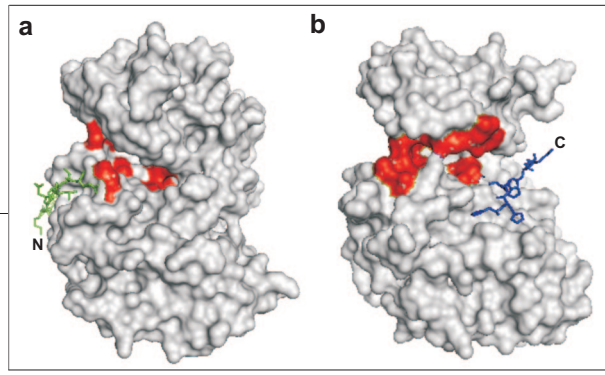


**Figure 2. Location of the docking peptide and substrate peptide when bound to the kinase. a) Model of ERK2 (PDB 2FYS) with a docking peptide (green) depicted in a stick form. The docking peptide, GIMLRRLQKGNLPVRAL, is derived from the D-domain of MAP kinase phosphatase 3 (*18*). The N terminus is marked with an "N". b) Model of CDK2 (PDB 1QMZ) with a substrate peptide (HHASPRK) depicted in a stick form (blue). The C terminus of the substrate peptide is labeled. In panels a and b, the ATP-interacting residues of the kinase are highlighted in red. All images were generated with Pymol.**

length of linker connecting the docking peptide and substrate peptide is challenging. If the linker is too short, it could hinder the binding of the two peptides to their respective docking sites, and if the linker is longer than optimal, it may inflict an energetic penalty on the ability of the two peptides to bind to ERK. Since the exposed surface of the ERK protein between the docking and substrate binding sites is composed of a mixture of neutral and acidic amino acids, repeating units of the neutral, hydrophilic molecule 8-mino-3,6-dioxaoctanoyl (AOO) were utilized as the linker between the substrate and docking peptides (Figure 1, panel b).

**Relative Orientation of the Subcomponents of the Designed Peptides.** Crystal structures of docking peptides bound to ERK show the docking peptides with an amino to carboxy orientation (Figure 2, panel a). While no structures are available of ERK bound to a substrate peptide, the structure of a closely related kinase, CDK2, bound to a peptide is available (Figure 2, panel b) (*43*). If the orientation of a peptide substrate bound to ERK is similar to that bound to CDK2, then the C-terminus of the docking peptide would extend from the N-terminus of the substrate when attached *via* an appropriate length linker (dock–linker–substrate). Surprisingly, another order for the connection of the substrate and docking peptides is supported in the literature. Data from Bardwell and colleagues (*29*) demonstrated that on average, a greater percentage of solid-support-bound substrate peptide (PLSP) was phosphorylated by ERK when a docking peptide was attached to the C-terminus of the peptide compared with substrate with an N-terminal docking peptide. Thus, a second order of the peptides is possible in which the N-terminus of the docking peptide could extend from the C-terminus of the substrate peptide when attached *via* a suitable linker (substrate–linker–dock).

**Description of the Designed ERK Substrates.** Since two orders were possible to bridge the docking peptide and substrate peptides, modular peptide substrates with each of the three selected docking sequences were synthesized in both orders (Table 1). From the crystallographic structures, the estimated maximal distance between the termini of the docking and substrate peptides was 30 Å. Three AOO repeats with an extended length

**TABLE 1. Designed peptide substrates for ERK**

| | Amino acid sequence | | |
|---|---|---|---|
| **Substrate name** | **Docking motif** | **Linker between peptides** | **Consensus motif** |
| ERKSub | | | TGPLSPGPF[c] |
| SCRAMMEK2 | LIPALRNMVRPKLTPA[a] | $(AOO)_3$[b] | TGPLSPGPF[c] |
| ELKERK | PQKGRKPRDLELPL[a] | $(AOO)_3$ | TGPLSPGPF[c] |
| MEK1ERK | MPKKKPTPIQLNP[a] | $(AOO)_3$ | TGPLSPGPF[c] |
| MEK2ERK | MLARRKPVLPALTINP[a] | $(AOO)_3$ | TGPLSPGPF[c] |
| STE7ERK | FQRKTLQRRNLKGLNLNL[a] | $(AOO)_3$ | TGPLSPGPF[c] |

| | Amino acid sequence | | |
|---|---|---|---|
| **Substrate name** | **Consensus motif** | **Linker between peptides** | **Docking motif** |
| ERKMEK1 | TGPLSPGPF[a] | $(AOO)_3$ | MPKKKPTPIQLNP[c] |
| ERKMEK2 | TGPLSPGPF[a] | $(AOO)_3$ | MLARRKPVLPALTINP[c] |
| ERKSTE7 | TGPLSPGPF[a] | $(AOO)_3$ | FQRKTLQRRNLKGLNLNL[c] |

[a]The amino terminus was covalently attached to fluorescein. [b](AOO) is the linker, 8-amino-3,6-dioxaocatanoyl, which connects the docking motif peptide to the consensus motif peptide. [c]The carboxy terminus was amidated.

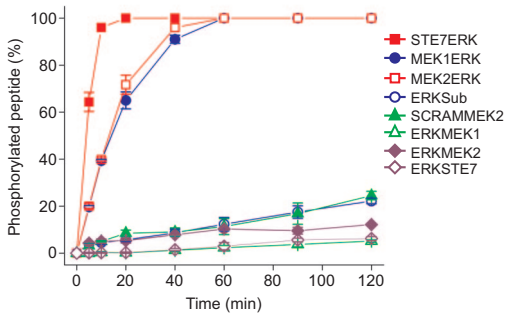


**Figure 3. Phosphorylation of the designed substrates by ERK1. The designed substrates (1 μM) were incubated with ERK1 in the presence of ATP and $Mg^{2+}$. Aliquots of the reaction mixture were removed at varying time points, and the amount of phosphorylated peptide was measured. The data points represent the average of three measurements, and the error bars indicate their standard deviation.**

of 30 Å were utilized as a linker between the docking and substrate peptides. Two control substrate peptides were also utilized for these studies (Table 1). The first peptide, referred to as ERKSub, was the substrate sequence, TGPLSPGPF, without a docking peptide. The second control peptide termed SCRAMMEK2 possessed a C-terminal substrate peptide. A linker with three AOO units was placed onto the N-terminus of the substrate. At the N-terminus of the linker was a peptide with the same amino acids as the docking peptide derived from MEK2 but with the amino acids in a randomized sequence.

**Phosphorylation of the Designed Substrates by ERK.** To determine whether the designed substrates were phosphorylated with greater efficiency than the substrate peptide alone, the peptides (1 μM) were incubated with ERK kinase in the presence of ATP and $Mg^{2+}$. At varying times, aliquots of the reaction mixtures were removed, and the amount of phosphorylated peptide was measured using laser-induced capillary electrophoresis, a fluorescence anisotropy assay, or both. The peptides fell into two clear groups, one in which all of the peptide was phosphorylated within 60 min and a second group in which <20% of the peptide was phosphorylated after 120 min (Figure 3). The first group with rapid phosphorylation was comprised entirely of the designed peptides with an N-terminal docking sequence. The group with the slower phosphorylation was composed of the peptides with a C-terminal docking peptide, the substrate alone, and the peptide with the N-terminal randomized docking sequence. The addition of any of the docking peptides to the N-terminus of the linker–substrate peptide dramatically enhanced phosphorylation of the modular substrate peptide. When the STE7 sequence was used as the docking peptide N-terminal to the substrate, the most rapid peptide phosphorylation was obtained with nearly 100% of the peptide phosphorylated in 10 min. The STE7 peptide potentiated the rate of phosphorylation more than either the MEK1 or MEK2 sequences. This result is consistent with existing data demonstrating that the protein STE7 possesses a higher affinity for mammalian ERK compared with either the MEK1 or the MEK2 protein (*28*). In contrast, addition of the docking peptides to the C-terminus of the substrate–linker peptide resulted in a similar or increased time for phosphorylation compared with the substrate alone. Since a C-terminal docking peptide was ineffective, regardless of intrinsic affinity, in enhancing phosphorylation, there was a clear preferred orientation for the docking peptide with respect to the substrate peptide. Because the control peptide SCRAMMEK2 was phosphorylated at similar rates compared with that of the substrate peptide alone, an amino acid composition (but not sequence) identical to that of a docking peptide was not sufficient to confer the enhanced rate of phosphorylation.

**Apparent $K_M$ ($K_M^{app}$) of the Designed Substrates for ERK.** The designed peptides possess two binding sites to the kinase. Steady-state kinetics were used to model the binding of the designed peptides to the kinase and subsequent phosphorylation and release of the peptide (Supplementary Scheme 1). The equation for the reaction velocity ($v$) *versus* the concentration of the docked substrate peptide ([D–S]) could be reduced to the form $v = V_{max}^{app}[D–S]/(K_M^{app} + [D–S])$ for $K_M^{app}$ defined as the apparent $K_M$ and $V_{max}^{app}$ defined as the apparent $V_{max}$. The reaction velocity at varying [D–S] was measured for each of the substrates and fit to the preceding equation to derive a $K_M^{app}$ and $V_{max}^{app}$ (Table 2). The $v$ *versus* [D–S] plots for all substrates irrespective of whether the docking peptide was on the N-terminus or C terminus were well fit by this equation (Figure 4). The substrates with N-terminal docking peptides from the MEK1, MEK2, and ELK proteins possessed a $K_M^{app}$ that was over 20-fold better than the $K_M$ of the substrate alone. Thus docking sequences from either up-stream kinases for ERK or down-stream substrates of ERK improved the

**TABLE 2. Reaction constants of the designed peptides for ERK**

| Peptide | $K_M$ or $K_M^{app}$ (μM) | $k_{cat}$ or $k_{cat}^{app}$ ($min^{-1}$) | $k_{cat}^{app}/K_M^{app}$ ($\mu M^{-1}\ min^{-1}$) |
|---|---|---|---|
| ERKSub | 127 ± 17 | 250 ± 20 | 1.9 |
| SCRAMMEK2 | 96 ± 36 | 130 ± 30 | 1.3 |
| ERKMEK1 | 344 ± 7.4 | 170 ± 10 | 0.5 |
| ERKMEK2 | 338 ± 73 | 180 ± 20 | 0.5 |
| ERKSTE7 | 173 ± 47 | 160 ± 70 | 1.0 |
| ELKERK | 4.4 ± 3.1 | 140 ± 20 | 32 |
| MEK1ERK | 3.7 ± 3.3 | 120 ± 40 | 32 |
| MEK2ERK | 5.6 ± 4.9 | 140 ± 60 | 25 |
| STE7ERK | 0.6 ± 0.4 | 170 ± 40 | 280 |

properties of the substrate peptide. The addition of the STE7 docking peptide to the substrate increased the $K_M^{app}$ of the substrate by >200-fold compared with the $K_M$ for ERKSub. Remarkably all of these N-terminal docking sequences conferred a similar or better affinity constant ($K_M^{app}$) to the substrate peptide compared with the $K_M$ of the full-length parent protein (from which the docking peptides were derived). These dramatic affinity enhancements suggested that the docking peptide dominated the on or off rates or both of the designed substrates for ERK. The substrate with the N-terminal randomized MEK2 sequence possessed a $K_M^{app}$ similar to the $K_M$ of the substrate alone again suggesting that the correct order of the amino acids in the docking peptide was critical for binding to ERK and not for turnover. The designed substrates with the C-terminal docking peptides all possessed poorer $K_M^{app}$ than the $K_M$ of the substrate peptide alone. The docking peptide in this instance may have compromised substrate access to the catalytic cleft of the kinase.

***$k_{cat}^{app}$* of the Designed Peptide Substrates for ERK.** The $k_{cat}^{app}$ for the designed substrates was calculated from the $V_{max}^{app}$ and the measured enzyme concentration (Table 2). The $k_{cat}^{app}$ of all of the designed substrates was decreased but within a factor of two of the $k_{cat}$ of the substrate alone. Neither the identity nor location (N-terminal or C terminal) of the docking peptide substantially influenced the $k_{cat}^{app}$. The substrate peptide attached to the randomized MEK2 sequence also possessed a similar $k_{cat}^{app}$ to that of the designed substrates with docking peptides. Thus, the decreased $k_{cat}^{app}$ of the designed peptides was most likely not a result of the binding of a docking peptide to ERK. In addition, the $k_{cat}^{app}$ of the designed peptides as well as ERKSub was similar to that reported for other peptide substrates for ERK (Supplementary Table 1). To further assess the effects of the docking peptide on the activity of the kinase, the rate of phosphorylation of ERKSub was measured in the presence and absence of free MEK1 docking peptide. The progress of the reaction was nearly identical for free docking peptide at concentrations between 0 and 300 μM (Figure 5). Thus, the binding of the docking peptide to ERK does not appear to modulate the activity of ERK as had been suggested in the literature (*44*). The small decrease in the $k_{cat}^{app}$ of the docked substrates relative to the $k_{cat}$ of the free substrate may be the result of steric hindrance created by the additional amino acid residues near the substrate. These results suggest that the substrate peptide rather than the docking peptide dominated the kinetics of the phosphate transfer.

**Apparent Efficiency of the Designed Peptide Substrates for ERK.** The apparent efficiency ($k_{cat}^{app}/K_M^{app}$) was calculated for each of the substrates in order to compare their suitability as substrates for ERK (Table 2). The efficiency of the designed substrates with the docking peptide on the C terminus and of the control substrate, SCRAMMEK2, was equivalent to or less than that of the substrate peptide alone. In contrast when the docking

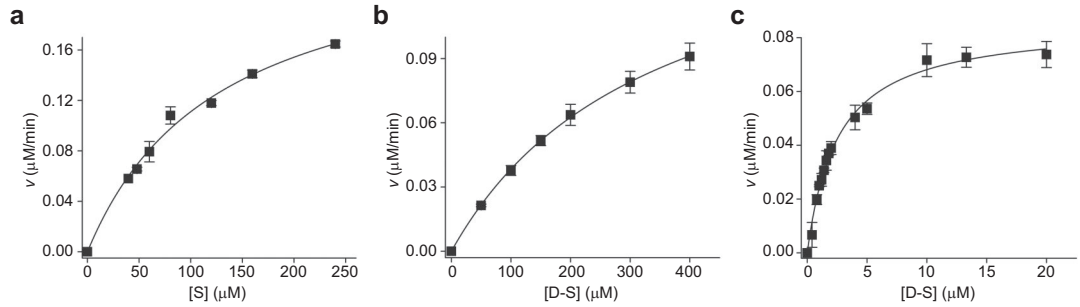


**Figure 4. The rates of phosphorylation of the designed substrates fit to an equation of the form $v = V_{max}^{app}[D–S]/(K_M^{app} + [D–S])$ where [D–S] is the concentration of the docked substrate peptide. a) Shown is the *v vs* substrate concentration ([S]) curve for ERKSub. b, c) Shown is the *v vs* docked substrate concentration ([D–S]) curve for ERKMEK1 (b) and MEK1ERK (c). The solid lines represent the fits to the Michaelis–Menten equation (panel a) or the equation above (panels b and c). The initial velocity *v* is reported per micromole of enzyme used.**

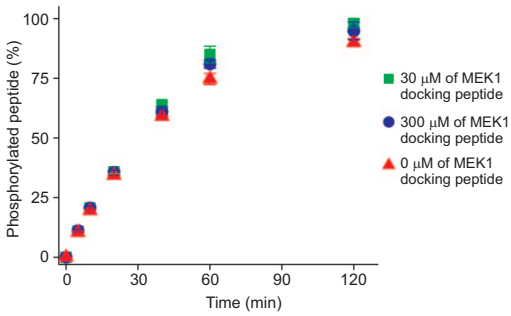


**Figure 5. Time course of ERKSub phosphorylation by ERK in the presence and absence of the free MEK1 docking peptide. The initial concentration of ERKSub was 150 μM. The data points represent the average of three measurements, while the error bars represent the standard deviation.**

peptides were placed at the N-terminus, the efficiency was improved by over an order of magnitude. Remarkably the STE7 docking peptide improved the efficiency by 150-fold. Compared with other peptide substrates for ERK reported in the literature, the $k_{cat}^{app}/K_M^{app}$ for STE7ERK was 1000-fold improved (Supplementary Table 1). Addition of a docking sequence can offer substantial improvements to the efficiency of a substrate peptide. A comparison of the efficiency of the designed peptide substrate with known protein substrates of ERK revealed that the apparent efficiency ($k_{cat}^{app}/K_M^{app}$) of the designed peptide STE7ERK is similar to or better than that of many protein substrates (Supplementary Table 2).

**Specificity of Designed Substrates for ERK.** ERKSub can be phosphorylated by p38α MAP kinase with a $K_M$ of 1200 ± 600 μM and $k_{cat}$ of 570 $min^{-1}$. To determine whether the designed substrates could also be phosphorylated by p38α, the reaction velocity was measured for varying concentrations of either MEK1ERK or MEK2ERK. As with the phosphorylation reaction with ERK, the plots of velocity *versus* substrate concentration were used to derive a $K_M^{app}$ and $k_{cat}^{app}$ of the designed substrates for p38α. Both the MEK1- and MEK2-derived docking peptides improved the $K_M^{app}$ for p38α (Table 3). However, the $K_M^{app}$ of the designed substrates for p38α remained an order of magnitude greater than that for ERK. Thus the designed substrates retained their greater affinity for ERK relative to p38α MAP kinase. The $k_{cat}^{app}$ of MEK1ERK and MEK2ERK for p38α kinase was not substantially improved relative to the $k_{cat}$ of p38α kinase for ERKSub. As with ERK, the docking peptide dominated the binding properties ($K_M^{app}$) of the designed substrates for p38α kinase but the substrate peptide dominated the rate of phosphorylation.

**TABLE 3. Reaction constants of the designed peptides for p38α**

| Peptide | $K_M$ or $K_M^{app}$ (μM) | $k_{cat}$ or $k_{cat}^{app}$ ($min^{-1}$) | $k_{cat}^{app}/K_M^{app}$ ($\mu M^{-1}$ $min^{-1}$) |
|---|---|---|---|
| ERKSub | 1200 ± 600 | 570 ± 185 | 0.5 |
| MEK1ERK | 65 ± 14 | 570 ± 28 | 8.7 |
| MEK2ERK | 30 ± 10 | 1000 ± 42 | 33 |

**Conclusion.** A peptide docking sequence derived from either a downstream substrate or an upstream activator was appended to an ERK substrate peptide to yield a high-efficiency substrate for ERK without loss of specificity. In addition to the MAP kinases, a multitude of other kinases rely on docking interactions with their protein substrates at sites that are distinct from the substrate's phosphoacceptor site. Protein tyrosine kinases such as ABL and Src possess SH2 domains that recognize a phosphotyrosine motif on their substrate (*45*). Peptides matching these motifs (pTyr-Glu-Glu-Ile for Src and pTyr-Asp-X-Pro for ABL) bind to the kinases with excellent affinity and thus are suitable candidates for linkage to substrate motifs. Protein kinase C and protein kinase A also incorporate interaction domains for binding partners, and peptides binding to these interaction domains might serve as tools to improve the efficiency of substrate peptides (*46*). Consequently, appending small docking peptides to kinase substrate motifs may be a general strategy by which simple, modular, yet high-efficiency substrates can be constructed for kinases.

## METHODS

**Materials.** Competent *Escherichia coli* cells (strain BL21 DE3) were obtained from Invitrogen. Ni–nitrilotriacetic acid (NTA) agarose columns were from Qiagen, while fused silica capillaries were obtained from Polymicro Technologies. All other reagents were from Sigma-Aldrich Chemical Co.

**Expression and Purification of Active ERK1 and p38α.** Competent *E. coli* cells (strain BL21 DE3, Invitrogen) were transformed

by electroporation in the presence of a plasmid containing the gene for ampicillin resistance, active MEK-1R4F, and the $His_6$-tagged ERK1 enzyme (pETH$is_6$MEK1 R4F+ERK1 plasmid). Expressed MEK-1R4F phosphorylates and activates ERK1 *in vivo* and the hexahistidine tag ($His_6$) aids in the purification of ERK1 (*47*). The transformed bacteria were plated on dishes containing Luria broth (LB) and ampicillin and incubated at 37 °C overnight. On day 2, 100 mL of Terrific broth (TB) containing ampicillin (100 $\mu g\ mL^{-1}$) was inoculated with a colony of the transformed bacteria, and the culture was incubated by shaking overnight at 37 °C. On day 3, 25 mL of the overnight culture was added to four flasks each containing 1 L of TB plus ampicillin (100 $\mu g\ mL^{-1}$). The cultures were grown at 30 °C and induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached 0.4. Cultures were then incubated for 12–16 h. The cells were harvested by centrifugation at 5000 × *g* for 10 min. The cell pellets were resuspended in cold sonication buffer (50 mM $NaPO_4$, 0.3 M NaCl, pH 8.0) containing the protease inhibitors leupeptin (1 $\mu g\ mL^{-1}$), antitrypsin (4 $\mu g\ mL^{-1}$), and 100 μM phenyl methyl sulfonyl fluoride (*47*). The cells were lysed in a French press (Thermo Electron Corp). The cell lysate was clarified by centrifugation at 10,000 × *g* for 60 min at 4 °C. The active ERK1 protein was purified using a Ni–NTA agarose column (Qiagen). The protein was dialyzed against buffer containing 12.5 mM HEPES (pH 7.3), 100 mM KCl, 6.25% (v/v) glycerol, and 0.5 mM DTT at 4 °C. After dialysis, the protein was concentrated by centrifugation through a filter (Centricon-10, Milllipore-Amicon) and aliquoted into volumes of 20 μL and stored at −70 °C. Active p38α kinase was expressed and purified in an identical fashion but using the plasmid pETH$is_6$/MEK6DD+p38α.

**Peptide Synthesis and Preparation.** Peptides labeled on the N-terminus with fluorescein and amidated on the C-terminus were synthesized and purified by Anaspec Inc. The molecular weight of the peptide was verified by mass spectroscopy. All peptides were dissolved in water, aliquoted, and stored at −70 °C. The concentration of the peptides was determined by performing amino acid analysis in the presence of a standard by the Molecular Structure Facility at the University of California in Davis (*48*).

**Measurement of Peptide Phosphorylation.** The immobilized metal ion affinity-based fluorescence polarization (IMAP) assay (Molecular Devices Corp.) was used to measure the amount of phosphorylated peptide in reaction mixtures (http://www.moleculardevices.com/pages/reagents/imap.html). The IMAP assay measures the change in anisotropy when a phosphorylated peptide binds to a metal nanoparticle. A calibration curve was constructed by measuring the anisotropy of solutions with known ratios of phosphorylated to nonphosphorylated peptide. The standard with 100% phosphorylated peptide was prepared using ERK1 kinase, and the percentage phosphorylation was verified by capillary electrophoresis(*40*) Anisotropy was measured using a fluorescence plate reader (SpectraMax M5, Molecular Devices) with an excitation of 485 nm (bandwidth of 9 nm) and emission of 525 nm (bandwidth of 15 nm).

**Kinase Assay Conditions.** Protein kinase assays were performed at 30 °C in assay buffer [10 mM Tris HCl (pH 7.2), 1 mM DTT, 0.01% Tween 20, and 0.05% $NaN_3$] with 10 mM $MgCl_2$, 1 mM ATP, and 1 nM ERK1 or 0.7 nM p38α kinase in a total reaction volume of 100 μL. Substrate concentrations ranged from 3 to 600 μM. Aliquots of 5 μL were removed from the reaction mixture at 5, 10, 20, 40, 60, and 120 min intervals. The reactions were stopped by boiling at 90 °C for 4 min. The amount of phosphorylation was measured using the IMAP assay as described above. In order to validate the IMAP assay some of the samples were also analyzed using capillary electrophoresis (Supporting Information).

**Determination of Reaction Constants.** The reaction velocity was determined from mixtures in which <10% of the substrate was consumed. The velocity was plotted against the substrate concentration. Fits to the data were performed using Origin 7.5 (OrginLab Corp.)

**Measurement of the Concentration of Active ERK1 and p38αMAP Kinase.** The concentration of active ERK1 was estimated by measuring the kinetic constants of the ERK substrate peptide, TGPLSPGPF, under the following conditions: 20 mM Tris (pH 8.0), 200 μM ATP, 1 mM DTT, 1 mM benzamidine, 10 mM $MgCl_2$ at 30 °C for which the peptide ERKtide (ATGPLSPGPFGRR) has kinetic parameters of $K_M = 450 \pm 230$ μM and $k_{cat} = 120 \pm 8\ min^{-1}$ for ERK2 (*35*). The activity of p38α MAP kinase was calculated by measuring the kinetic constants of the Cantley peptide (Fl-GIPTSPITTTYFFFKKK) under the following conditions: 0.1 M HEPES (pH 7.6), 1 mM ATP, 10 mM $MgCl_2$, 10% glycerol, 30 °C) for which it has the reported kinetic parameters $K_M = 103 \pm 16$ μM and $k_{cat} = 22.8 \pm 1.2\ s^{-1}$ (*50*).

*Acknowledgment:* We gratefully acknowledge M. Cobb (University of Texas Southwestern) for supplying us with the plasmids (pETH$is_6$MEK1 R4F+ERK1 and pETH$is_6$/MEK6DD+p38α) for expression of active ERK and p38α MAP kinases. We would also like to thank B. Liao (University of California, Irvine) for assistance with expression of active ERK. We also thank L. Bardwell (University of California, Irvine) for assistance in selection of the docking peptides for ERK. This work was supported by NSF and NIH.

*Supporting Information Available:* This material is free of charge *via* the Internet.

## REFERENCES

1. Cobb, M. H. (1999) MAP kinase pathways, *Prog. Biophys. Mol. Biol. 71*, 479–500.
2. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) Signal transduction through MAP kinase cascades, *Adv. Cancer Res. 74*, 49–139.
3. Bradham, C., and McClay, D. R. (2006) p38 MAPK in development and cancer, *Cell Cycle 5*, 824–828.
4. Kaminska, B. (2005) MAPK signalling pathways as molecular targets for anti-inflammatory therapy—from molecular mechanisms to therapeutic benefits, *Biochim. Biophys. Acta (BBA) - Proteins Proteomics 1754*, 253–262.
5. Manning, A. M., and Davis, R. J. (2003) Targeting JNK for therapeutic benefit: from junk to gold? *Nat. Rev. Drug Discovery 2*, 554–565.
6. Nottage, M., and Siu, L. L. (2002) Rationale for Ras and raf-kinase as a target for cancer therapeutics, *Curr. Pharm. Des. 8*, 2231–42.
7. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., and Bottomley, W. (2002) Mutations of the BRAF gene in human cancer, *Nature 417*, 949–954.
8. Marshall, C. J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase, *Curr. Opin. Genet. Dev. 4*, 82–89.
9. Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992) Erks: their fifteen minutes has arrived, *Cell Growth Differ. 3*, 135–142.
10. Whitmarsh, A. J., and Davis, R. J. (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways, *J. Mol. Med. 74*, 589–607.
11. Karin, M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases, *J. Biol. Chem. 270*, 16483–16486.
12. Davis, R. J. (1993) The mitogen-activated protein kinase signal transduction pathway, *J. Biol. Chem. 268*, 14553–14556.
13. Cobb, M. H., Hepler, J. E., Cheng, M., and Robbins, D. (1994) The mitogen-activated protein kinases, ERK1 and ERK2, *Semin. Cancer Biol. 5*, 261–268.

14. Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase), *EMBO J. 10*, 885–892.
15. Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. J. (2003) Activation mechanism of the MAP Kinase ERK2 by dual phosphorylation, *Curr. Biol. 13*, 1145–1149.
16. Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, E. J. (1994) Atomic structure of the MAP kinase ERK 2 at 2.3 Å resolution, *Nature 367*, 704–711.
17. Songyang, Z., Lu, K. P., Kwon, Y. T., Tsai, L. H., Filhol, O., Cochet, C., Brickey, D. A., Soderling, T. R., Bartleson, C., and Graves, D. J. (1996) A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1, *Mol. Cell. Biol. 16*, 6486–6493.
18. Liu, S., Sun, J. P., Zhou, B., and Zhang, Z. Y. (2006) Structural basis of docking interactions between ERK2 and MAP kinase phosphatase 3, *Proc. Natl. Acad. Sci. U.S.A. 103*, 5326–5331.
19. Rainey, M. A., Callaway, K., Barnes, R., Wilson, B., and Dalby, K. N. (2005) Proximity-induced catalysis by the protein kinase ERK2, *J. Am. Chem. Soc. 127*, 10494–10495.
20. Chang, C. I., Xu, B. E., Akella, R., Cobb, M. H., and Goldsmith, E. J. (2002) Crystal structures of MAP kinase p38 complexed to the docking sites on its nuclear substrate MEF2A and activator MKK3b, *Mol. Cell 9*, 1241–1249.
21. Jacobs, D., Glossip, D., Xing, H., Muslin, A. J., and Kornfeld, K. (1999) Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase, *Genes Dev. 13*, 163–175.
22. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators, *Nat. Cell Biol. 2*, 110–116.
23. Yang, S. H., Yates, P. R., Whitmarsh, A. J., Davis, R. J., and Sharrocks, A. D. (1998) The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif, *Mol. Cell. Biol. 18*, 710–720.
24. Tanoue, T., Maeda, R., Adachi, M., and Nishida, E. (2001) Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions, *EMBO J. 20*, 466–479.
25. Enslen, H., Brancho, D. M., and Davis, R. J. (2000) Molecular determinants that mediate selective activation of p38 MAP kinase isoforms, *EMBO J. 19*, 1301–1311.
26. Sharrocks, A. D., Yang, S. H., and Galanis, A. (2000) Docking domains and substrate-specificity determination for MAP kinases, *Trends Biochem. Sci. 25*, 448–453.
27. Lee, T., Hoofnagle, A. N., Kabuyama, Y., Stroud, J., Min, X., Goldsmith, E. J., Chen, L., Resing, K. A., and Ahn, N. G. (2004) Docking motif interactions in MAP kinases revealed by hydrogen exchange mass spectrometry, *Mol. Cell 14*, 43–55.
28. Bardwell, A. J., Flatauer, L. J., Matsukuma, K., Thorner, J., and Bardwell, L. (2001) A conserved docking site in MEKs mediates high-affinity binding to MAP kinases and cooperates with a scaffold protein to enhance signal transmission, *J. Biol. Chem. 276*, 10374–10386.
29. Bardwell, A. J., Abdollahi, M., and Bardwell, L. (2003) Docking sites on mitogen-activated protein kinase (MAPK) kinases, MAPK phosphatases and the Elk-1 transcription factor compete for MAPK binding and are crucial for enzymic activity, *Biochem. J. 370*, 1077–1085.
30. Smith, J. A., Poteet-Smith, C. E., Malarkey, K., and Sturgill, T. W. (1999) Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK *in vivo*, *J. Biol. Chem. 274*, 2893–2898.
31. Seidel, J. J., and Graves, B. J. (2002) An ERK2 docking site in the Pointed domain distinguishes a subset of ETS transcription factors, *Genes Dev. 16*, 127–137.
32. Kallunki, T., Su, B., Tsigelny, I., Sluss, H. K., Derijard, B., Moore, G., Davis, R., and Karin, M. (1994) JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation, *Genes Dev. 8*, 2996–3007.
33. Adler, V., Franklin, C. C., and Kraft, A. S. (1992) Phorbol esters stimulate the phosphorylation of c-jun but not v-jun: regulation by the N-terminal delta domain, *Proc. Natl. Acad. Sci. U.S.A. 89*, 5341–5345.
34. Tanoue, T., and Nishida, E. (2003) Molecular recognitions in the MAP kinase cascades, *Cell. Signalling 15*, 455–462.
35. Robinson, M. J., Harkins, P. C., Zhang, J., Baer, R., Haycock, J. W., Cobb, M. H., and Goldsmith, E. J. (1996) Mutation of position 52 in ERK2 creates a nonproductive binding mode for adenosine 5-triphosphate, *Biochemistry 35*, 5641–5646.
36. Haycock, J. W. (2002) Peptide substrates for ERK1/2: structure-function studies of serine 31 in tyrosine hydroxylase, *J. Neurosci. Methods 116*, 29–34.
37. Giepmans, B. N., Adams, S. R. Ellisman. M. H. and Tsien, R. Y. (2006) The fluorescent toolbox for assessing protein localization and function. *Science 312,* 217–224.
38. Sato, M., Kawai, Y., and Umezawa, Y. (2007) Genetically encoded fluorescent indicators to visualize protein phosphorylation by extracellular signal regulated kinase in single living cells, *Anal. Chem. 79*, 2570–2575.
39. Sharma, V., Agnes, R. S., and Lawrence, D. S. (2007) Deep quench: an expanded dynamic range for protein kinase sensors, *J. Am. Chem. Soc. 129*, 2742–2743.
40. Meredith, G., Sims, C. E., Soughayer, J. S., and Allbritton, N. L. (2000) Measurement of kinase activation in single mammalian cells, *Nat. Biotechnol. 18*, 309–312.
41. Clark-Lewis, I., Sanghera, J. S., and Pelech, S. L. (1991) Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase, *J. Biol. Chem. 266*, 15180–15184.
42. Xu, B., Wilsbacher, J. L., Collisson, T., and Cobb, M. H. (1999) The N-terminal ERK-binding site of MEK1 is required for efficient feedback phosphorylation by ERK2 *in vitro* and ERK activation *in vivo*., *J. Biol. Chem. 274*, 34029–34035.
43. Brown, N. R., Noble, M. E. M., Endicott, J. A., and Johnson, L. N. (1999) The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases, *Nat. Cell Biol. 1*, 438–443.
44. Zhou, T., Sun, L., Humphreys, J., and Goldsmith, E. J. (2006) Docking interactions induce exposure of activation loop in the MAP kinase ERK2, *Structure 14*, 1011–1019.
45. Pawson, T., Gish, G. D., and Nash, P. (2001) SH2 domains, interaction modules and cellular wiring, *Trends Cell Biol. 11*, 504–511.
46. Ubersax, J. A., and Ferrell, J. E., Jr. (2007) Mechanisms of specificity in protein phosphorylation, *Nat. Rev. Mol. Cell. Biol. 8*, 530–541.
47. Khokhlatchev, A., Xu, S., English, J., Wu, P., Schaefer, E., and Cobb, M. H. (1997) Reconstitution of mitogen-activated protein kinase phosphorylation cascades in bacteria. Efficient synthesis of active protein kinases, *J. Biol. Chem. 272*, 11057–11062.
48. Cooper, C., Packer, N., and Williams, K. (2001) Amino Acid Analysis Protocols, in *Methods in Molecular Biology*, Vol. 159, Humana Press, Totawa, New Jersey.
50. Chen, G., Porter, M. D., Bristol, J. R., Fitzgibbon, M. J., and Pazhanisamy, S. (2000) Kinetic mechanism of the p38-MAP kinase: phosphoryl transfer to synthetic peptides, *Biochemistry 39*, 2079–2087.