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Use of Docking Peptides to Design Modular Substrates with High Efficiency for MAP Kinase ERK

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

[†] Department of Chemical Engineering and Materials Science, University of California, Irvine, CA 92697

[‡] Department of Chemistry, University of California, Irvine, CA 92697

* Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599

Abstract

The mitogen-activated protein (MAP) 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.

INTRODUCTION

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 proteins 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 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 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 $(\text{Arg/Lys})_{2-3}(\text{X})_{1-6}\text{-}\phi_{\text{A}}\text{-X-}\phi_{\text{B}}$; where ϕ_{A} and ϕ_{B} are hydrophobic residues such as Leu, Ile, Val 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 which are similar 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 α 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 which 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 ($K_{\text{M}} > 100 \mu\text{M}$) for ERK compared to 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 incorporates a peptide substrate sequence between two fluorescent proteins and adjacent to a phosphopeptide-binding motif (37). Others methods use phosphorylation of a

peptide-based substrate to assist in reassembly of between a circularly permuted 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 used by Allbritton and colleagues 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 (40).

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* using an oriented, degenerate, peptide library (17). 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 Pro 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 “ATGPLSPGPFGR” for ERK2 are $450 \pm 230 \mu\text{M}$ and $120 \pm 8 \text{ min}^{-1}$ 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 (¹MPKKKPTPIQLNP¹³) was chosen since MEK1 binds ERK2 with an affinity of $29 \mu\text{M}$ (28). A docking peptide derived from the N-terminal 16 amino acids of MEK2 (¹MLARRKPVLPALTINP¹⁶) was utilized as a potential intermediate-affinity peptide since MEK2 possesses an affinity of $9 \mu\text{M}$ for ERK2 (28). STE7, a MAP kinase kinase, from the yeast *Saccharomyces cerevisiae* interacts with mammalian ERK1 and ERK2 with a K_D of $1 \mu\text{M}$. Thus a peptide comprised of the N-terminal 18 amino acids (²FQRKTLQRRNLKGLN¹⁹) was employed as a potential, higher-affinity binding peptide than the peptide derived from MEK2 (28). A fourth, docking peptide, ³¹⁰PQKGRKPRDLELPL³²³ 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 $5 \mu\text{M}$ (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 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-amino-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 1, 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 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 to substrate with an N-terminal docking peptide (29). 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 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 ERK_{sub} was the substrate sequence, TGPLSPGPF, without a docking peptide. The second control peptide termed SCRAMMEK2 possessed a C-terminal, substrate peptide. A linker with 3 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 Mg²⁺. At varying times, aliquots of the reaction mixtures were removed and the amount of phosphorylated peptide was measured using laser-induced capillary electrophoresis and/or a fluorescence anisotropy assay. 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 less than 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 to either the MEK1 and MEK2 proteins (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 to 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 to 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) vs. the concentration of the docked substrate peptide ($[D-S]$) could be reduced to the form: $v = V_{\text{max}}^{\text{app}} * [D-S] / (K_M^{\text{app}} + [D-S])$ for K_M^{app} defined as the apparent K_M and $V_{\text{max}}^{\text{app}}$ defined as the apparent V_{max} . The reaction velocity at varying $[D-S]$ was measured for each of the substrates and fit to the above equation to derive a K_M^{app} and $V_{\text{max}}^{\text{app}}$ (Table 2). The v vs. $[D-S]$ 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 twenty-fold better than the K_M of the substrate alone. Thus docking sequences from either upstream kinases for ERK or downstream substrates of ERK improved the properties of the substrate peptide. The addition of the STE7 docking peptide to the substrate increased the K_M^{app} of the substrate by over 200 fold compared to 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 to the K_M of the full-length parent protein (from which the docking peptide were derived). These dramatic affinity enhancements suggested that the docking peptide dominated the on and/or off rates 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.

$k_{\text{cat}}^{\text{app}}$ of the Designed Peptide Substrates for ERK

The $k_{\text{cat}}^{\text{app}}$ for the designed substrates was calculated from the $V_{\text{max}}^{\text{app}}$ and the measured enzyme concentration (Table 2). The $k_{\text{cat}}^{\text{app}}$ 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 $k_{\text{cat}}^{\text{app}}$. The substrate peptide attached to the randomized MEK2 sequence also possessed a similar $k_{\text{cat}}^{\text{app}}$ to that of the designed substrates with docking peptides. Thus, the decreased $k_{\text{cat}}^{\text{app}}$ of the designed peptides was most likely not a result of the binding of a docking peptide to ERK. In addition, the $k_{\text{cat}}^{\text{app}}$ 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 $k_{\text{cat}}^{\text{app}}$ 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 ($k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$) 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 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 to other peptide substrates for ERK reported in the literature, the $k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$ 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 ($k_{\text{cat}}^{\text{app}}/K_{\text{M}}^{\text{app}}$) 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 p38 α MAP kinase with a K_{M} of $1200 + 600 \mu\text{M}$ and k_{cat} of 570 min^{-1} . To determine whether the designed substrates could also be phosphorylated by p38 α , the reaction velocity was measured for varying concentrations of either MEK1ERK or MEK2ERK. As with the phosphorylation reaction with ERK, the plots of velocity vs. substrate concentration were used to derive a $K_{\text{M}}^{\text{app}}$ and $k_{\text{cat}}^{\text{app}}$ of the designed substrates for p38 α . Both the MEK1 and MEK2-derived docking peptides improved the $K_{\text{M}}^{\text{app}}$ for p38 α (Table 3). However the $K_{\text{M}}^{\text{app}}$ of the designed substrates for p38 α remained an order of magnitude greater than that for ERK. Thus the designed substrates retained their greater affinity for ERK relative to p38 α MAP kinase. The $k_{\text{cat}}^{\text{app}}$ of MEK1ERK and MEK2ERK for p38 α kinase was not substantially improved relative to the k_{cat} of p38 α kinase for ERKsub. As with ERK, the docking peptide dominated the binding properties ($K_{\text{M}}^{\text{app}}$) of the designed substrates for p38 α kinase but the substrate peptide dominated the rate of phosphorylation.

Conclusion

A peptide docking sequence derived from either a downstream substrate or 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 which are distinct from the substrate's phospho-acceptor site. Protein tyrosine kinases such as ABL and Src possess SH2 domains which 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 *E coli* cells (strain BL21 DE3) were obtained from Invitrogen. Ni-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 p38 α

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 His₆ tagged ERK1 enzyme (pETHis₆MEK1 R4F+ERK1 plasmid). Expressed MEK-1R4F phosphorylates and activates ERK1 *in vivo* and the hexahistidine tag (His₆) 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 $\mu\text{g mL}^{-1}$) 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 4 flasks each containing 1 L of TB plus ampicillin (100 $\mu\text{g mL}^{-1}$). The cultures were grown at 30 °C and induced with 0.3 mM isopropyl- β -D-thio galactopyranoside (IPTG) when the optical density at 600 nm reached 0.4. Cultures were then incubated for 12–16 hrs. The cells were harvested by centrifugation at 5000 x g for 10 min. The cell pellets were resuspended in cold sonication buffer (50 mM NaPO₄, 0.3 M NaCl, pH 8.0) containing the protease inhibitors, leupeptin (1 $\mu\text{g mL}^{-1}$), anti-trypsin (4 $\mu\text{g mL}^{-1}$) 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 x 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, Millipore-Amicon) and aliquoted into volumes of 20 μL and stored at -70 °C. Active p38 α kinase was expressed and purified in an identical fashion but using the plasmid, pETHis₆/MEK6DD+p38 α .

Peptide Synthesis and Preparation

Peptides labeled on the amino terminus with fluorescein and amidated on the carboxy 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 (49). 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 non phosphorylated 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% NaN₃] with 10 mM MgCl₂ and 1 mM ATP and 1nM ERK1 or 0.7nM p38 α kinase in a total reaction volume of 100 μ L. Substrate concentrations ranged from 3–600 μ M. Aliquots of 5 μ l were removed from the reaction mixture at 5, 10, 20, 40 and 60, and 120 minute intervals. The reactions were stopped by boiling at 90 °C for 4 mins. 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 (Supplementary information).

Determination of Reaction Constants

The reaction velocity was determined from mixtures in which less than 10% of the substrate was consumed. The velocity was plotted against the substrate concentration. Fits to the data were performed using Origin 7.5 (OriginLab Corp.)

Measurement of the Concentration of Active ERK1 and p38 α MAP 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 μ M ATP, 1 mM DTT, 1 mM benzamidine, 10 mM MgCl₂ at 30 °C for which the peptide ERKtide (ATGPLSPGPFGR) has kinetic parameters of $K_M = 450 \pm 230 \mu\text{M}$, $k_{\text{cat}} = 120 \pm 8 \text{ min}^{-1}$ for ERK2 (35). The activity of p38 α MAP kinase was calculated by measuring the kinetic constants of the Cantley peptide (FI-GIPTSPITTTYFFFKKK) under the following conditions, 0.1 M HEPES (pH 7.6), 1 mM ATP, 10 mM MgCl₂, 10% glycerol, 30 °C for which it has the reported kinetic parameters, $K_M = 103 \pm 16 \mu\text{M}$, $k_{\text{cat}} = 22.8 \pm 1.2 \text{ s}^{-1}$ (50)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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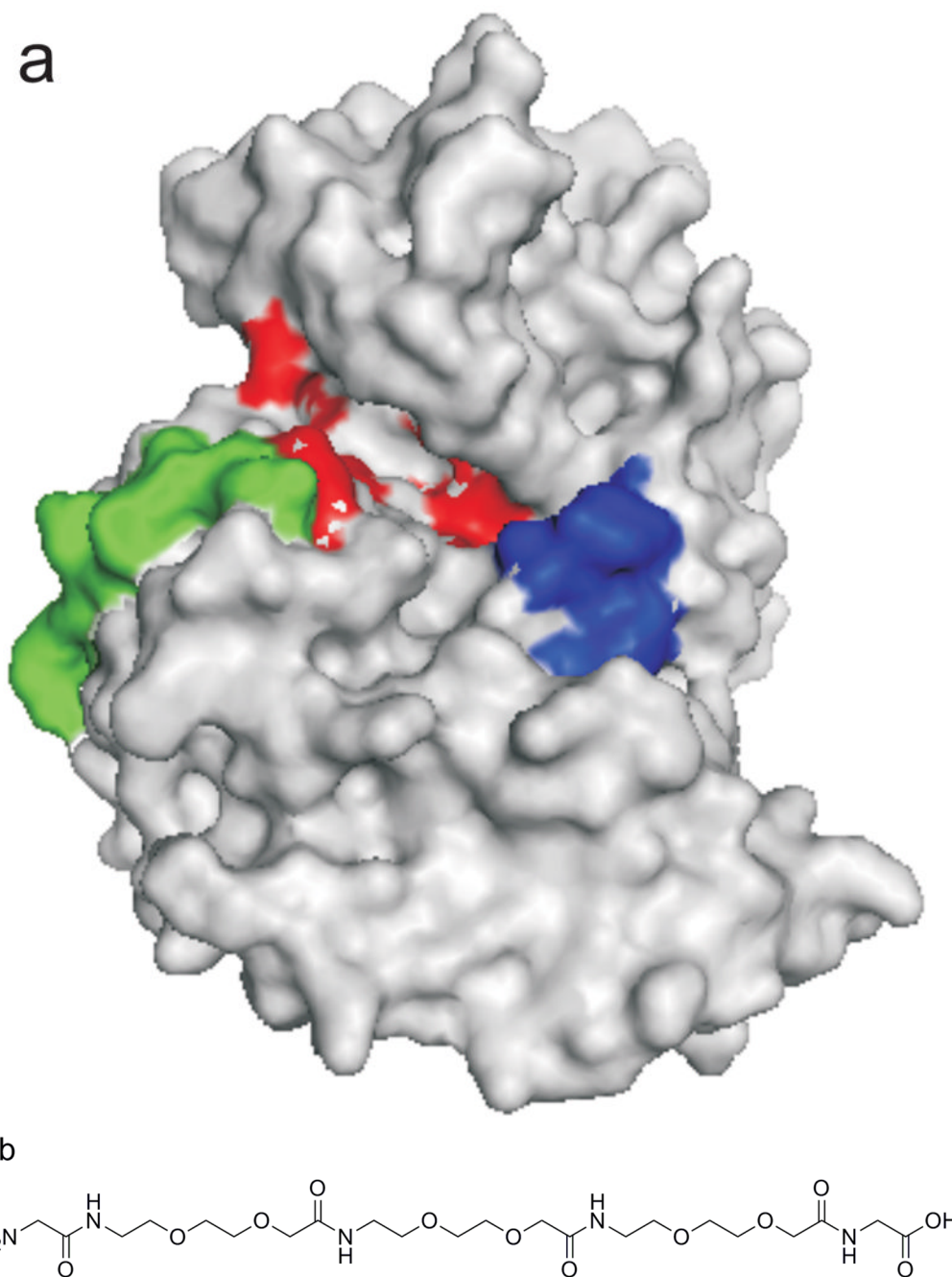


Figure 1. A linker is required to connect the docking peptide to the substrate sequence. a) Structure of ERK2 (PDB 2FYS) indicating the residues interacting with the substrate D domain (green), substrate sequence (blue), and ATP (red). The image was created with Pymol (DeLano Scientific LLC). b) Shown is the linker AOO₃ with a glycine at both termini (Gly-(AAO)₃-Gly).

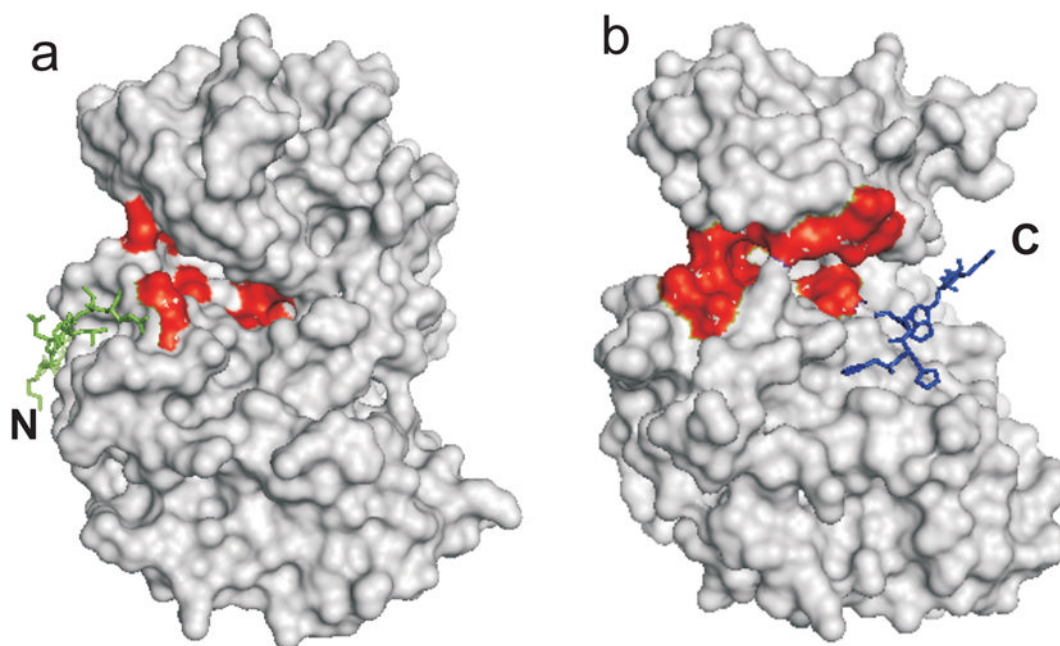


Figure 2.

Location of the docking peptide and substrate peptide when bound to the kinase. a) Model of ERK2 (PDB 2FY5) 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 a) and b), the ATP-interacting residues of the kinase are highlighted in red. All images were generated with Pymol.

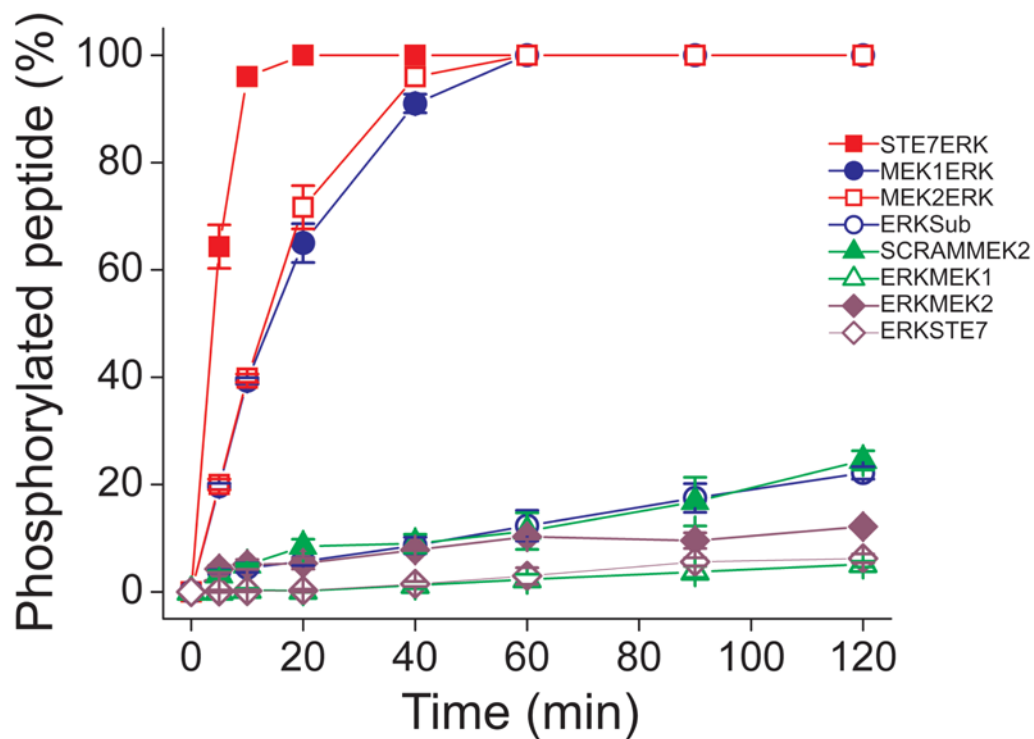
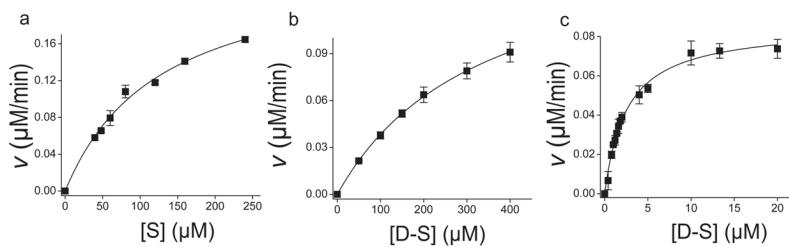


Figure 3. Phosphorylation of the designed substrates by ERK1. The designed substrates ($1\mu\text{M}$) were incubated with ERK1 in the presence of ATP and Mg^{2+} . Aliquots of the reaction mixture were removed at varying time points and the amount of phosphorylated peptide was measured. The data points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

**Figure 4.**

The rates of phosphorylation of the designed substrates fit to an equation of the form $v = V_{\max}^{\text{app}} * [D-S] / (K_M^{\text{app}} + [D-S])$ where $[D-S]$ is the concentration of the docked substrate peptide. a) Shown is the v vs substrate concentration ($[S]$) curve for ERKSub. b) and 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 (a) or the equation above (b and c). The initial velocity v is reported per μmole of enzyme used.

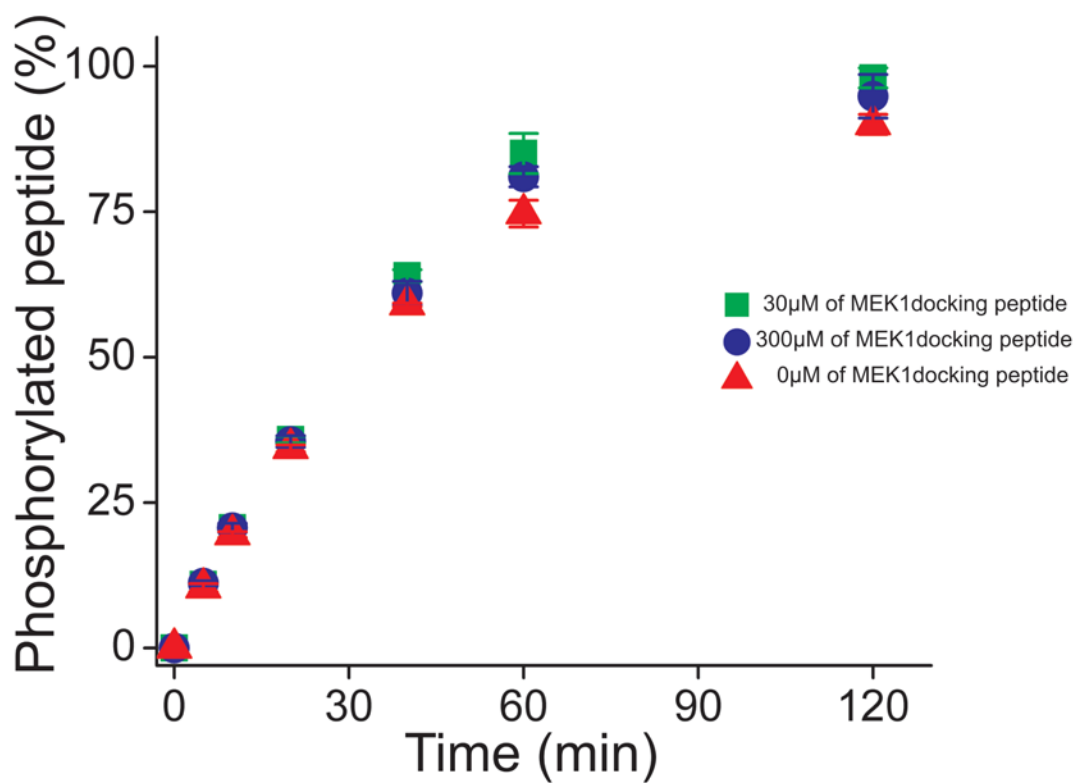


Figure 5. Time course of ERKSub phosphorylation by ERK in the presence and absence of the free MEK1 docking peptide. The initial concentration of ERKSub was 150 μM. The data points represent the average of three measurements while the error bars represent the standard deviation.

Table 1

Designed peptide substrates for ERK

Substrate Name	Docking motif	Amino acid sequence Linker Between Peptides	Consensus motif
ERKSub			TGPLSPGPF ^c
SCRAMMEK2	^a LIPALRNMVRPKLTPA	^b (AOO) ₃	TGPLSPGPF ^c
ELKERK	^a PQKGRKPRDLELPL	(AOO) ₃	TGPLSPGPF ^c
MEK1ERK	^a MPKKKPTPIQLNP	(AOO) ₃	TGPLSPGPF ^c
MEK2ERK	^a MLARRKPVLPALTINP	(AOO) ₃	TGPLSPGPF ^c
STE7ERK	^a FQRKTLQRRNLKGLNLNL	(AOO) ₃	TGPLSPGPF ^c
	<i>Consensus motif</i>	<i>Linker Between Peptides</i>	<i>Docking motif</i>
ERKMEK1	^a TGPLSPGPF	(AOO) ₃	MPKKKPTPIQLNP ^c
ERKMEK2	^a TGPLSPGPF	(AOO) ₃	MLARRKPVLPALTINP ^c
ERKSTE7	^a TGPLSPGPF	(AOO) ₃	FQRKTLQRRNLKGLNLNL ^c

^aThe amino terminus was covalently attached to fluorescein

^b(AOO) is the linker, 8-amino-3,6-dioxoacatanoyl which connects the docking motif peptide to the consensus motif peptide

^cThe carboxy terminus was amidated.

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 \pm 17	250 \pm 20	1.9
SCRAMMEK2	96 \pm 36	130 \pm 30	1.3
ERKMEK1	344 \pm 7.4	170 \pm 10	0.5
ERKMEK2	338 \pm 73	180 \pm 20	0.5
ERKSTE7	173 \pm 47	160 \pm 70	1.0
ELKERK	4.4 \pm 3.1	140 \pm 20	32
MEK1ERK	3.7 \pm 3.3	120 \pm 40	32
MEK2ERK	5.6 \pm 4.9	140 \pm 60	25
STE7ERK	0.6 \pm 0.4	170 \pm 40	280

Table 3Reaction constants of the designed peptides for p38 α

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