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Phage display identifies novel peptides that bind extracellular-regulated protein kinase 2 to compete with transcription factor binding†

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ABSTRACT: Extracellular-regulated protein kinase 2 (ERK2) is a serine/threonine-specific protein kinase capable of phosphorylating multiple protein substrates within a cell. In an attempt to identify novel peptides that bind and inhibit the function of an active form of ERK2, phage display was carried out using a disulfide-constrained peptide library ($X_2CX_{14}CX_2$). Several phage clones were identified by an enzyme-linked immunosorbent assay (ELISA) that competed with both a protein substrate and adenosine triphosphate (ATP) for immobilized ERK2. A chemically synthesized peptide derived from these experiments, NH_2-KKKIRCIRGWTKDIRTLADSCQY-COOH, inhibited ERK2 phosphorylation of the protein substrate EtsΔ138, exhibiting competitive and mixed inhibition towards EtsΔ138 and $MgATP^{2-}$, respectively. Surprisingly, the same peptide displayed equally potent inhibition towards the phosphorylation of ATF2 by p38 MAPKα, another MAP kinase that has $\sim$46% sequence similarity to ERK2. This study indicates that active ERK2 can be targeted by phage display to find novel antagonists to kinase function and suggests that protein-binding sites within the MAPK family may contain conserved features that render them susceptible to ligand binding. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: phage display; ERK2; MAPK; peptide; inhibitor

INTRODUCTION

ERK2 is a member of the mitogen-activated protein kinase (MAPK) signaling cascade that is conserved among eukaryotes. Following mitogen or growth factor activation of cell surface receptors, an internal cell-signaling pathway is stimulated which ultimately leads to gene transcriptional changes. Activation of the MAPK signaling cascade involves Raf kinase activation which phosphorylates and activates MAPK kinases (MAPKKs) by dual phosphorylation. Activated MAPKKs are capable of phosphorylating their cognate MAPKs. Upon phosphorylation of both Thr-183 and Tyr-185 in the flexible phosphorylation loop, ERK2 undergoes a conformational change leading to its activation,[1] homodimerization and nuclear translocation.[2] The covalent phosphate additions maintain the enzyme in an active state until phosphatase-mediated hydrolysis of one or both phosphates leads to ERK2 inactivation. Activated ERK2 is capable of regulating gene transcription by phosphorylating transcription factors in response to mitogens. These events lead to phenotypic changes at the cellular level including proliferation, differentiation and cell growth.

Since the activation of ERK2 stimulates cells to proliferate, many cancers and transforming agents utilize the

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Abbreviations: ERK2, extracellular-regulated protein kinase 2; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; pIII, protein III; SA, specific activity; LB, Luria broth; TB, Terrific broth; TU, transduced units; Tris–HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol with hydrochloric acid; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; ABTS, 2,2′-azine di(3-ethylbenzthiazolinesulfonate); ESI, electrospray ionization; EtsΔ138, murine (His_6-tagged)Ets-$1^{(1–138)}$; DTT, dithiothreitol; BSA, bovine serum albumin fraction V; ATP, adenosine triphosphate; KCl, potassium chloride; b-ERK2, biotin-ERK2; PMSF, phenyl methyl sulfonyl fluoride; TPCK, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; GST, glutathione *S*-transferase; PEG, polyethylene glycol 8000; MALDI, matrix-assisted laser desorption/ionization; CDK, cyclin-dependent kinase; ATF2, activating transcription factor 2; UDHB, underdehydrated hydrogen bond.

MAPK cell-signaling pathway to carry out unregulated cell growth.[3] Therefore, ERK2 is an obvious target for the development of small molecule inhibitors for cancer therapeutics. However, development of antagonists specific to individual members of the protein kinase family has proven difficult, owing to the conservation of active site residues in the hydrophobic $MgATP^{2-}$ binding pocket. Many inhibitors of protein kinases target the $MgATP^{2-}$ binding site as seen in the case of polyhydroxylated flavones,[4] isoquinolinesulfonamides,[5,6] and both pyridinylimidazoles and triarylimidazoles,[7–9] which inhibit cyclin-dependent kinase (CDK), cAMP-dependent protein kinase and p38 MAPKα, respectively. Unfortunately, these inhibitors often bind other enzymes that utilize $MgATP^{2-}$,[10] and must compete with high intracellular concentrations of ATP (3.2 ± 1.7 mM).[11] An alternative approach to kinase inhibition involves the use of peptides that resemble a consensus phosphorylation site that is recognized by an enzyme's active site.[12] Active site-directed peptide inhibitors, however, are not typically potent due to weak binding affinities and since several kinases tend to recognize similar phosphorylatable regions, they may also be fairly non-specific. For example, both MAPKs and CDKs phosphorylate serine or threonine residues followed by a proline so a peptide inhibitor designed to inhibit a MAPK may also inhibit a CDK. A recent approach physically linked ATPγS and a peptide inhibitor, both capable of binding the enzyme independently, to generate bisubstrate inhibitors in the nanomolar range.[13]

Another potentially more promising approach towards the inhibition of certain protein kinases is to block protein binding sites on the enzyme that lie distant from the active site. Some protein kinases, including members of the MAPK[15] and CDK[14] family, contain docking regions for proteins located outside of the active site. In the case of the MAPKs, these regions regulate the specificity of interactions of the kinase with protein substrates, and also with positive and negative regulators of their activity. The C-terminal region of ERK2, for example, contains acidic residues Asp-316 and Asp-319, that function as the docking site for the activator MAPKK1, the inactivator MAPK phosphatase 3 and several protein substrates including Elk-1, MAPK signal-integrating kinase 1 and ribosomal S6 kinase.[15,16] Therefore, these putative docking regions are attractive drug targets in that they may contain primary and/or secondary structure information specific to an individual kinase and its interactions with other proteins. Indeed, peptides with an RXL motif have been shown to disrupt substrate binding to CDK2 through such a mechanism.[17]

Proteins that contain 'hot-spots,' such as human growth hormone,[18] integrins,[19] and streptavidin,[20] have evolved to bind small peptide motifs tightly. Hot spots are proposed to be regions where intramolecular backbone hydrogen bonds are not sufficiently dehydrated. These bonds have been termed underdehydrated hydrogen bonds (UDHBs).[21] Thus, a UDHB on the surface of the protein may indicate a potential binding site, or hot spot, that can associate with proteins or peptides to exclude water from backbone hydrogen bonds. Such hot spots have been elucidated using peptide phage display.[22] Phage display is a technique used to select phage that bind a protein of interest (referred to as a receptor of phage binding) from a large library of phage, each expressing a novel peptide on their protein coat. Each peptide is displayed as a fusion protein on the extracellular surface of the phage coat and can potentially be captured by binding to the receptor protein. Each peptide is physically linked to its internal phage DNA, allowing rapid identification. Using this technique, phage particles with a phenotype of interest can be selected through receptor binding and their genotype determined.

Previously, Zwick *et al.* used phage display to select peptides that bind ERK1/2 MAP kinase. However, a peptide substrate (TGPLSPGPF) was used to elute the phage competitively, presumably targeting only the phage that bind the active site.[23] The selected sequences found to bind ERK1/2 in their study, FHKPLKR and NPAHSPW, were not further characterized for their ability to bind or inhibit ERK1/2.

In this study, phage display was performed to select for novel peptides that bind an activated form of ERK2 using a disulfide-constrained peptide library fused to the phage minor coat protein III (pIII) using multiple rounds of affinity purification (biopanning).[22]

EXPERIMENTAL

Materials

The $X_2CX_{14}CX_2$ fd-tet phage library, inserted into a fUSE5 virion, was a gift from B. Wang (Case Western Reserve University, Cleveland, OH, USA) and the *Escherichia coli* K91BluKan strain used for propagation of the phage was a gift from G. Smith (Washington University, St. Louis, MO, USA). Tween-20 was obtained from J. T. Baker (Phillipsburg, NJ, USA). Sulfo-NHS-LC-biotin, biotin, streptavidin-coated 96-well plates, antibodies and ELISA reagents were purchased from Pierce (Rockford, IL, USA). Kinase assays utilized Na_4ATP (Roche, Indianapolis, IN, USA) and [γ-^{32}P]ATP^{4-} (ICN, Irvine, CA, USA). BSA was obtained from Fisher Scientific (Fair Lawn, NJ, USA), DTT from USBio (Swampscott, MA, USA), Tris base from EM Industries (Gibbstown, NJ USA) and all other reagents from Sigma (St. Louis, MO, USA). Proteins were concentrated using Centricon-10 centrifugation filter devices with a molecular weight cutoff of 10 kDa (Millipore, Bedford, MA, USA).

Purification of His_6- and GST-tagged proteins

DNA encoding rat-His_6-ERK2 and MAPKK1 R4F were simultaneously expressed in *Escherichia coli* BL21

(DE3) pLysS giving rise to the activated (dual-phosphate) form of ERK2 and purified essentially as described,[2] dialyzed overnight at 4 °C into buffer S0 (20 mM HEPES, pH 7.3, 100 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 2 mM DTT) containing 10% glycerol, concentrated using a Centricon-10 filter and snap-frozen in liquid nitrogen.

DNA encoding murine-His_6-EtsΔ138 in a pET28a vector (Invitrogen) was expressed and purified as described previously;[24] however, DNaseI was omitted from the lysis buffer and the Mono Q HR 10/10-purified EtsΔ138 was dialyzed overnight into S0 buffer lacking DTT at 4 °C, concentrated to 25 mg ml^{-1} and snap-frozen.

DNA encoding inactive rat-His_6-p38 MAPKα in a pET14b vector (Invitrogen) was expressed in *E. coli* BL21 (DE3) pLysS, grown in LB containing 50 µg ml^{-1} ampicillin to an OD_{600} 0.6–0.8 at 30 °C and induced for protein expression with 0.5 mM IPTG for 3 h. The His_6-tagged protein was purified using Ni-NTA affinity chromatography as described,[24] dialyzed overnight in S0 buffer containing 10% glycerol at 4 °C, concentrated to 2 mg ml^{-1} and snap-frozen.

DNA encoding the mutant GST-MAPKK6b (S207E/T211E) fusion protein was expressed in *E. coli* BL21 (DE3) pLysS, grown to an OD_{600} of 0.6–0.8 at 30 °C in LB containing 50 µg ml^{-1} ampicillin, and induced with 0.5 mM IPTG for 3 h. The cells (9 g from 3.2 l) were centrifuged at 6000 rpm at 4 °C in a GS3 rotor (Sorvall) and the pellet was snap-frozen. The cells were thawed, resuspended in 50 mM Tris–HCl, pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM PMSF, 0.1 mM TPCK, 1 mM benzamidine, 2 mM EDTA, 2 mM EGTA, 1% (w/v) Triton X-100, 0.25 M NaCl, and sonicated on ice (5 × 30 s pulses) to maintain a temperature lower than 4 °C. The cellular debris was pelleted by spinning at 16 000 rpm for 15 min at 4 °C in an SS34 rotor (Sorvall). The supernatant was gently mixed for 2 h at 4 °C with 2 ml of glutathione agarose resin to bind the fusion protein. The agarose beads were washed with 30 ml of 50 mM Tris–HCl, pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM PMSF, 0.1 mM TPCK, 1 mM benzamidine and 0.125 M NaCl. The fusion protein was eluted with 10 ml of 20 mM reduced glutathione dissolved in 50 mM Tris–HCl, pH 8.0, 0.1% BME, 0.1 mM PMSF, 0.1 mM TPCK and 1 mM benzamidine to yield 1.4 mg of total protein. The GST-MKK6b fusion protein was dialyzed at 4 °C in buffer S0 containing 10% glycerol, concentrated to 2 mg ml^{-1} and snap-frozen.

DNA encoding GST-ATF2-(1–115) was expressed and purified as described,[25] omitting protease inhibitors from the Mono Q buffers and dialyzed into S0 buffer lacking DTT at 4 °C.

Activation of p38 MAPKα

p38 MAPKα (4 µM) and a constitutively active mutant form of its activator (GST-MAPKK6b) (0.3 µM) were incubated with an activation buffer containing 10 mM HEPES, pH 8.0, 4 mM ATP, 20 mM $MgCl_2$ and 2 mM DTT for 3 h to phosphorylate p38 MAPKα. Activated p38 MAPKα was purified using methods similar to activated ERK2[26] and eluted from the Mono Q HR 10/10 at 0.42 M NaCl. The eluted protein was dialyzed overnight in buffer S0 containing 10% glycerol at 4 °C, concentrated to 2 mg ml^{-1} and snap-frozen. An analogous reaction was carried out in the presence of [γ-^{32}P]ATP (150 cpm $pmol^{-1}$) to determine the molar ratio of phosphate incorporated into p38 MAPKα. This was carried out by spotting portions of the reaction on P81 cellulose paper[26] or TCA precipitated, washed to remove radiolabeled ATP, counted in a scintillation counter, and determined as 2.8 mol mol^{-1} phosphate (only 2 mol mol^{-1} is expected for active p38 MAPKα).

Biotinylation of ERK2

Biotin was covalently linked to active ERK2 to form biotin-ERK2 (b-ERK2) using sulfosuccinimidyl-6-(biotinamido)-hexanoate (sulfo-NHS-LC-biotin). The NHS mixed carbonate reacts with primary amine side-chains to form a stable carbamate bond with ERK2. A 2:1 mol mol^{-1} ratio of NHS-LC-biotin to ERK2 was added in buffer S0 containing 1 mM ATP on ice for 2 h. ATP was added to protect biotin incorporation into Lys-52 of ERK2, which is required for efficient catalysis.[27] Unreacted biotin was removed using a Centricon-10 filter by buffer- exchange (at least 3 × 2 ml of buffer S0 containing 10% glycerol). Aliquots were snap-frozen in liquid nitrogen and stored at − 80 °C. Biotinylation was confirmed by Western blot analysis using a horseradish-peroxidase conjugated secondary rabbit anti-goat antibody (1:10 000) and primary goat anti-biotin antibody (1:10 000) as described by the manufacturer (Pierce). ERK2 that was not biotinylated was not detected in the Western blot.

Kinetic measurements of biotin-ERK2 in solution

The specific activity of ERK2 was determined by measuring initial rates of radiolabeled phosphate incorporation into the substrate EtsΔ138 by spotting the reaction on P81 paper as previously described.[24] Reactions were performed in a 100 µl volume in the presence of 1–2 nM b-ERK2, 11.3 µM EtsΔ138, 10 mM $MgCl_2$, 100 µg ml^{-1} BSA, and 100 µM [γ-^{32}P]ATP (500–1000 cpm $pmol^{-1}$) in buffer S0 lacking DTT at 25 °C.

Kinetic measurements of immobilized b-ERK2

Immobilization of b-ERK2 in 96-well streptavidin-coated plates was carried out by adding the enzyme in binding buffer B0 (buffer S0 containing 0.1% 2-mercaptoethanol substituted for DTT) for 2 h at 4 °C with mild

shaking. The wells were washed once with buffer B0 to remove the unbound b-ERK2, prior to the addition of blocking buffer B1 (buffer B0 containing 5 mg ml^{-1} BSA and 0.1 μg ml^{-1} streptavidin, to bind any contaminating biotin in the BSA) for 1 h at 4 °C. The wells were washed (6 × 5 min) with buffer S0 lacking DTT to remove residual 2-mercaptoethanol in preparation for non-reducing biopanning conditions. Immobilized b-ERK2 assays were carried out in the streptavidin-coated wells using similar conditions to the kinetic assays described above without addition of ERK2, since it is already immobilized. The number of active b-ERK2 molecules was estimated using the observed rate constant (k_{obs}) of immobilized b-ERK2 and the specific activity (SA) of b-ERK2 in solution using the equation

$$\text{Active immobilized b-ERK2 (mg)} = \frac{k_{obs}\ \text{immobilized (nmol min}^{-1}\text{)}}{\text{SA solution (nmol min}^{-1}\text{ mg}^{-1}\text{)}} \quad (1)$$

Large-scale amplification and purification of phage

K91BluKan cells were grown to late log phase at 37 °C in 200 ml of Terrific broth (TB)[28] containing 100 μg ml^{-1} kanamycin. Fd-tet bacteriophage were added after slow shaking for 5 min to allow F-pili regeneration. Slow shaking was continued to allow infection for 15 min. The cultures were added to 2 l of LB containing 0.22 μg ml^{-1} tetracycline and shaken at 300 rpm for 35 min. The concentration of tetracycline was increased to 18 μg ml^{-1} and shaken overnight. Purification of phage was carried out as described[29] preceding the cesium chloride purification steps that remove trace amounts of PEG. The purified phage were raised in TBS (25 mM Tris–HCl, pH 7.5, 3 mM KCl, 137 mM NaCl) containing 15% glycerol and snap-frozen in liquid nitrogen. To determine the quantity of phage, phage were titered using serial dilutions in TBS and allowed to infect late log phase K91BluKan cells at 25 °C for 10 min, followed by a 30 min incubation at 37 °C in 100 μl of TB containing 0.2 μg ml^{-1} tetracycline, plated on agar plates containing 40 μg ml^{-1} tetracycline and 100 μg ml^{-1} kanamycin, and grown overnight to select for phage-infected *E. coli*. The number of transduced units (TU) were determined by counting the colonies that gained resistance to tetracycline via bacteriophage infection.[29]

Biopanning conditions

Similar procedures to those described above were carried out to immobilize b-ERK2. After blocking with BSA, the wells were briefly washed six times with phage binding buffer B2 (TBS containing 5 mg ml^{-1} BSA). The phage were added in buffer B2 and allowed to bind immobilized b-ERK2 for 1 h (Rounds I–III) or 20 min (Round IV) at 25 °C with mild shaking. After phage binding in the first two rounds, 0.1 mM biotin was added for 5 min to free any streptavidin-binding phage. The wells were washed 12 times with buffer B2 containing 0.1% Tween-20 in the first two rounds, increasing to 0.3% Tween-20 in the third and fourth rounds. Washes were carried out for 1 min in Round I, extended to 10 min in Round II and 5 min in Round III and IV. Phage were eluted for 10 min using 100 μl of 0.2 M glycine, pH 2.2, containing 1 mg ml^{-1} BSA and immediately buffered with 15 μl of 1 M Tris–HCl, pH 9.1, following the elution. To prepare phage for freezing, 15 μl of 100% glycerol were added prior to storage at −80 °C. After each round of biopanning, eluted phage were titered and amplified for further rounds using phage eluted from wells containing 4 μg, 1 μg and 10 ng of plated b-ERK2 following Rounds I, II and III, respectively.

Enzyme-linked immunosorbent assay (ELISA)

Individual phage were obtained by growing a single transduced unit (colony) overnight in 3 ml of LB containing 40 μg ml^{-1} tetracycline and 100 μg ml^{-1} kanamycin. The phage were purified in 1.5 ml centrifuge tubes by subjecting 1200 μl of cleared supernatant to two overnight pecipitations at 4 °C by adding 200 μl of 16.7% PEG 8000 and 3.3 M sodium chloride followed by extensive mixing. The precipitated phage were raised in 100 μl of TBS containing 10% glycerol, snap-frozen and stored at −80 °C. Yields were ∼1 × 10^8 TU μl^{-1}. Similar conditions to biopanning were carried out by plating 500 ng of b-ERK2 and using ∼1 × 10^8 TU of an individually purified phage clone for binding b-ERK2 in buffer B2 for 1 h at 25 °C. Following phage binding, the wells were washed (12 × 5 min) with buffer B2 containing 0.1% Tween-20. Phage bound to immobilized b-ERK2 were detected by horseradish peroxidase-conjugated anti-M13 (1:5000 dilution in buffer B2 + 0.5% Tween-20) using the hydrogen donor immunopure ABTS to reduce hydrogen peroxide as described by the supplier (Pierce). The colorometric assay was monitored using a 96-well plate reader (Bio-Tek Instruments, Winooski, VT, USA) following the absorbance at 405 nm. ELISAs using 10 mM DTT to reduce cyclic phage peptides were carried out by adding the DTT to buffer B2 during phage binding. ELISAs using EtsΔ138 to compete with phage binding were carried out by adding EtsΔ138 in buffer S0 without DTT to buffer B2 for 10 min prior to phage addition. ELISAs using $MgATP^{2-}$ to compete with phage binding were carried out by adding $MgATP^{2-}$ in buffer B2 10 min prior to phage addition in buffer B2.

Synthesis and purification of peptide 20a

The peptide NH_2-KKKIRCIRGWTKDIRTLADSCQY-COOH (peptide 20a) was synthesized using Fmoc-based

solid-phase synthesis. The crude form of the peptide was dissolved in equilibration buffer [0.1% (v/v) trifluoroacetic acid and 5% (v/v) acetonitrile] and purified by reversed phase chromatography using an Econosil C18 10 μm column (Alltech, Deerfield, IL, USA), developed with a linear acetonitrile gradient of 5–80% over 75 min using a flow-rate of 5.0 ml min^{-1}. Fractions absorbing at 280 nm were applied to MALDI and analyzed for peptide purity. Pure fractions were pooled and analyzed by ESI and determined to have a molecular weight of 2782.6 Da.

Oxidation of peptide 20a

Pure linear peptide 20a was oxidized to form an intramolecular disulfide bond between the cysteine residues using iodine essentially as described.[30] The peptide was oxidized while stirring in 80% acetic acid by dropwise addition of iodine (in 100% acetic acid) until a pale yellow color appeared, indicating the completion of the reaction and allowed to react for 10 further min at 25 °C. The reaction was quenched with two volumes of water and the iodine was extracted six times with equal volumes of dichloromethane. The aqueous phase, containing the peptide, was lyophilized and re-purified using reversed phase chromatography (as described above). Analysis with ESI showed a shift to 2780.4 Da in accordance with the cyclization of the peptide and loss of two protons. The peptide was >90% pure as determined by reversed phase HPLC analysis. The concentration of peptide was determined by denaturation in 6 M guanidine chloride ($\varepsilon = 7210\ M^{-1}\ cm^{-1}$).[31]

Peptide inhibition

Inhibition studies were carried out using the active form of ERK2 and p38 MAPKα as described previously by measurement of radiolabeled phosphate incorporation into protein substrates EtsΔ138 and GST-ATF2-(1–115), respectively,[25,26] in the presence and absence of peptide 20a. All assays were carried out at 27 °C without a reducing agent in S1 buffer (20 mM HEPES, pH 7.3, 100 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 20 mM $MgCl_2$). ERK2/EtsΔ138 assays were carried out with 1 nM ERK2 and varied peptide 20a (0–50 μM) using either (a) varied EtsΔ138 (6.25–200 μM) and fixed ATP (270 μM or 2 mM in separate experiments) or (b) fixed EtsΔ138 (25 μM) and varied ATP (62.5–2000 μM). p38 MAPKα assays were performed in S1 buffer containing 4 nM p38 MAPKα, varied GST-ATF2-(1–115) (3–15 μM), fixed ATP (2 mM) and varied peptide 20a (0–20 μM). Concentrations of all proteins were determined using either Bradford assay[32] or 6 M guanidine hydrochloride denaturation.[31]

RESULTS AND DISCUSSION

Immobilized biotinylated ERK2 is active

The active form of ERK2 was purified, biotinylated and captured in streptavidin-coated wells via high-affinity biotin–streptavidin interactions to obtain a solid-state receptor for phage display biopanning. ERK2 was biotinylated using NHS-LC-biotin, which covalently attached biotin to surface-exposed primary amine groups. Biotinylation was confirmed by Western blot analysis using an antibody that specifically recognized biotin (data not shown). The specific activity of b-ERK2 'in solution' was determined (1925 nmol min^{-1} mg^{-1} using subsaturating substrate conditions as described in the Experimental section) by measuring initial rates of phosphate incorporation into a model protein substrate Ets-$1^{(1-138)}$ (EtsΔ138). The observed catalytic rate constant (k_{obs}) of b-ERK2 was within 2-fold of unbiotinylated ERK2 (data not shown), indicating that biotinylation did not greatly affect the activity of the enzyme.

b-ERK2 was immobilized in streptavidin-coated wells using conditions similar to biopanning and assayed for its ability to phosphorylate EtsΔ138 to determine the k_{obs} of immobilized b-ERK2. After plating 1 μg of the receptor b-ERK2 for immobilization on the plates, blocking and washing, to remove unbound b-ERK2 and BSA, immobilized b-ERK2 was assayed and shown to be catalytically active towards the transcription factor EtsΔ138 (k_{obs} immobilized: 0.007 nmol min^{-1}). Using Eqn (1), $\sim 5 \times 10^{10}$ molecules of immobilized b-ERK2 were estimated to be active following the initial stages of biopanning prior to phage addition. The activity associated with immobilized b-ERK2 phage receptor increased in a dose-dependent manner with respect to the amount of receptor plated (data not shown) indicating that increased concentrations of receptor led to increased b-ERK2 capture in streptavidin-coated wells. These results further confirm the biotinylation of b-ERK2 and demonstrate that streptavidin is capable of capturing the catalytically active receptor using biopanning conditions prior to the addition of phage. These data also indicate that immobilized b-ERK2 maintains functional protein binding sites, as indicated by its ability to phosphorylate a protein substrate, suggesting that b-ERK2 is an excellent receptor for phage displaying peptide targets.

Biopanning scheme for selection of phage that bind b-ERK2

Biopanning was carried out to select for phage that bind b-ERK2 from a large library of fd-tet bacteriophage displaying a single polypeptide ($X_2CX_{14}CX_2$) fused to the N-terminus of the surface coat protein pIII.[33] When phage are produced in *E. coli*, the N-terminus of pIII is exposed to the oxidized environment of the periplasm

causing disulfide bridge formation in the phage-displayed peptides containing cysteines, thereby, maintaining conformational rigidity of the peptides favored for binding.[34,35] Each round of biopanning was carried out as follows: (1) b-ERK2 was immobilized in streptavidin-coated wells; (2) non-specific binding sites were blocked using BSA; (3) the phage were allowed to bind the receptor; (4) the wells were washed to remove weak binding phage; and (5) the bound phage were eluted. Following each round, the eluted phage were amplified in *E. coli* to create multiple copies of each selected member so that they could be re-introduced to subsequent rounds of selection with increased selection pressures to encourage the selection of tight binding phage over the weaker binders. The stringency of selection was increased using methods such as extending the washing times following phage binding (to encourage the release of phage with fast k_{off} rates prior to the elution of those with slow k_{off} rates), lowering the concentration of receptor used to capture the phage (to select for tight binding phage *via* competition for rare receptor), decreasing the phage binding time (to select for those phage with higher k_{on} rates) and increasing the concentration of the detergent in the washing steps (to discourage weak and non-specific binding). Individual phage selected in later rounds were tested for their ability to bind b-ERK2 in a phage ELISA to select specifically for receptor-binding phage over non-specific binding that may be selected for by binding other members in the phage library or the streptavidin-coated wells. The genomic DNA of each receptor-binding phage was purified and sequenced to determine the primary amino acid sequence of the polypeptide displayed on the phage surface.

Round I: non-stringent biopanning conditions to select phage that bind b-ERK2

It is critical in early rounds to use non-stringent conditions that allow the phage an opportunity to bind the receptor since each member of the library is represented by only a few copies and the yields of tight binding phage are usually $<1\%$.[29] In later rounds, the selected phage are amplified several-fold so members are not as scarce, and the stringency of selection can be increased to select for tighter binding phage. Therefore, 4 μg of receptor were allowed to bind the streptavidin-coated plates, biopanned in the presence of 1.4×10^{11} TU of input phage in the first round of biopanning. These conditions allowed for a better than 3:1 ratio of phage to b-ERK2 (these data are based on the results that $\sim 5 \times 10^{10}$ molecules of b-ERK2 were able to phosphorylate EtsΔ138 during biopanning conditions after plating 1 μg of b-ERK2 on the plates. This ratio is based on TU. The physical phage particle ratio is estimated to be 5–20-fold larger than the TU ratio owing to the inherent low copy number of fd-tet phage.[36]), giving each of the phage a sufficient opportunity to bind b-ERK2. Eluted phage titers from wells containing b-ERK2 were 1.5-fold higher (Table 1) than those eluted from wells lacking the receptor, indicating that the presence of b-ERK2 increases phage capture. However, these results indicate that a significant portion of the eluted phage were binding non-specifically to the wells lacking receptor (non-specific binders are estimated from the number of phage eluted from wells lacking receptor). Therefore, the eluted phage from the receptor-containing well were amplified and biopanned in Round II to discriminate against the non-specific binders and select for phage members that bound specifically to b-ERK2.

Round II: extending wash time to select for small k_{off} rates

In the second round, the stringency of selection was increased by temporally extending the washes following phage binding to enhance selection of phage with small k_{off} values. Also, several concentrations of the receptor were biopanned under identical conditions to determine the effects of receptor concentration on phage binding. Eluted phage were shown to increase with increasing receptor concentrations, indicating that members of the Round II phage pool could specifically bind b-ERK2 and that increased receptor enhanced the binding opportunities for the phage (Fig. 1). These results are consistent with immobilized b-ERK2 phosphorylation assays of EtsΔ138 that exhibited an increase in phosphorylation of EtsΔ138 with respect to receptor concentration (data not shown). The enrichment ratio, defined as the number of eluted phage from wells containing receptor divided by the number of phage eluted from wells lacking receptor in a given round,[37] increased in Round II to 63-fold above non-specific binding compared with 1.5-fold in Round I (Table 1). Enrichment ratios above unity indicate that the phage are binding to the receptor. The increase in enrichment ratio is expected in early rounds and indicates an increase in selectivity for the receptor. It should also be noted that the number of non-specific phage binding to wells lacking the receptor also increased compared with

Table 1. Yields of eluted phage from biopanning experiments used for subsequent rounds[a]

Round	Plated b-ERK2 (ng)	Input phage (TU)	Eluted phage (TU)	Background (TU)
1	4000	7.9×10^{10}	2.3×10^{5}	1.5×10^{5}
2	1000	1.4×10^{11}	6.9×10^{7}	1.1×10^{6}
3	10	2.0×10^{11}	6.0×10^{5}	1.9×10^{5}
4	0.1	2.0×10^{10}	1.1×10^{4}	4.0×10^{3}

[a] In the first round, an amplified version of the original $X_2CX_{14}CX_2$ library was used. Thereafter, eluted phage were amplified from wells containing the amount of plated receptor concentration shown here and added as input phage to subsequent rounds. The concentrations of phage (TU) were determined by titering experiments based on the number of bacterial infections from eluted phage (see Experimental).

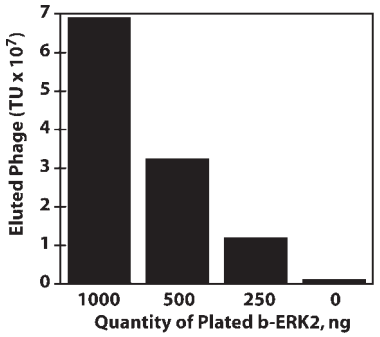


Figure 1. Eluted phage increase with increased receptor concentration. Biopanning was carried out using various amounts of plated b-ERK2 while holding the input phage constant at 1.4×10^{11} TU. Phage were allowed to bind for 1 h, washed (12×5 min) in buffer B2 containing 0.1% Tween-20 and eluted with 0.2 M glycine, pH 2.2, containing 1 mg ml^{-1} BSA. The eluted phage were equilibrated with Tris–HCl buffer and titered as described in the Experimental section

Round I, albeit at a lesser extent than those phage eluted from wells containing receptor. These results can be explained by the fact that both b-ERK2-binding phage and non-specific binding phage were selected for in Round I and amplified prior to Round II. To discourage the selection of non-specific phage in later rounds, the amplified pool of phage should contain a large ratio of receptor-binding to non-specific binding phage to increase the chance of selecting receptor-binding phage. Therefore, the phage eluted from wells using 1000 ng of plated receptor were amplified for further selection.

Round III: decreased receptor concentration to encourage phage competition

The stringency of selection was increased in Round III by using multiple receptor concentrations during biopanning and selecting the phage eluted from wells containing b-ERK2 with titers just above non-specific phage binding.[38] As receptor concentrations are lowered and become limiting, phage must compete with one another for limited receptor sites creating conditions where tighter binders can be selected for. In addition, the detergent concentration was increased to 0.3% Tween-20 during washes to discriminate further against non-specific binding. The concentrations of receptor allowed to bind the wells were 100, 10, 1 and 0 ng of b-ERK2. In wells in which 1 ng of receptor was plated, eluted phage titers were only ~2.8-fold above non-specific binding. Single bacteriophage were selected, amplified, purified and subjected to an ELISA to select for phage binding specifically to b-ERK2 (a positive signal in the presence of receptor and no signal in the absence of receptor). Of 40 phage clones tested, 11 tested positive for receptor binding, which is consistent with the fact that eluted phage from this well was ~2.8-fold above background (~14 expected). Single-stranded genomic DNA was purified from these clones and sequenced. Three of the sequences were similar, indicating that the selected clone bearing the peptide IRCIRGWTKDIRTLADSCQY was being selected for more often than others. No other sequences were identical with one another and a consensus sequence was not observed. The lack of consensus sequence is possibly due to the complexity of the library, since all possible combinations of amino acids cannot be represented in libraries greater than X_{6-7}. However, it could also be due to the fact that ERK2 recognizes and binds a variety of amino acid combinations, where individual amino acids make a small contribution to binding. Single phage clones eluted from wells containing more receptor (100 ng of plated b-ERK2) showed that 17/20 were able to bind b-ERK2 in an ELISA, further indicating that phage selected from wells containing more receptor are more likely to bind b-ERK2 due to a greater ratio of b-ERK2-binders to non-specific binders.

Round IV: decreased receptor

A fourth round of biopanning was carried out to determine if a consensus sequence could be reached or to confirm the selection of similar phage from the previous round. Receptor concentrations of 100, 10, 1, 0.1 and 0 ng were plated and biopanned. Individual phage eluted from wells in which 0.1 ng of receptor was plated were subjected to an ELISA and phage with the ability to bind b-ERK2 were sequenced. Six of the 13 positive phage clones contained the peptide sequence IRCIRGWTKDIRTLADSCQY (termed hereafter peptide 20), also seen in Round III (Table 2), indicating that the selection of phage bearing peptide 20 was favored under these conditions. Only one other sequence, HQCDSLHYPTSGWWATACHG (Table 2), was similar to those selected in Round III, indicating that both displayed peptides were selected under the conditions of Rounds III and IV.

Table 2. Round IV ERK2-binding peptides displayed on phage[a]

Clone	Amino acid sequence
12	IWCNGGGMTNGLLDAVRCHS
20[b,c]	IRCIRGWTKDIRTLADSCQY
32[b]	HQCDSLHYPTSGWWATACHG
40	QHCQGIWKSCSKHSTKCCSL
52	TGCRTTWIRWGWGQAETCTS
55	YSCKAWNRTVSGRIASGCLW
68	MGCVRRVTRPGLAVAEACSV
76	VFCRDVTRTPFGSFAPQCMR

[a] Isolated phage clones selected in Round IV were tested using a phage ELISA for the ability to bind b-ERK2. The DNA of positive clones was purified and sequenced to determine the primary amino acid sequence of the displayed peptide on the phage.
[b] Sequence also represented in phage after Round III (data not shown).
[c] Selected at a frequency 6 times greater than the others.

DTT inhibition of cyclic peptide binding

To determine whether the disulfide bond cyclization of the peptides on the phage were important for ERK2 binding, a reducing agent, DTT, was added to an ELISA using the phage clones selected from Round IV. An ELISA detects the ability of a population of an individual phage to bind the receptor. The ELISA was carried out in a manner similar to biopanning; however, instead of eluting the receptor-bound phage, a horseradish peroxidase-linked antibody specific for the phage coat was used to detect the relative amount of receptor-bound phage using a colorometric assay based on the reduction of hydrogen peroxide. Addition of DTT during phage binding significantly reduced the ELISA signal for all of the phage clones, indicating that the reduction of intramolecular disulfide bonds in each of the phage polypeptides ameliorates binding to b-ERK2 (Fig. 2).[35] These results indicate that the disulfide constraint within the displayed peptide is critical for efficient binding to b-ERK2 and also emphasizes the importance of biopanning in non-reducing conditions when using disulfide-constrained phage libraries.

EtsΔ138 disrupts phage binding to b-ERK2

The effects of protein–protein interactions on phage binding to b-ERK2 were studied using the protein substrate EtsΔ138 to elute phage during biopanning and to inhibit phage binding using a competition ELISA. Using Round III phage, biopanning conditions were carried out using EtsΔ138 to elute phage in lieu of acidic conditions (Fig. 3). These results show that phage can be eluted from b-ERK2 using a protein substrate EtsΔ138. We hypothesize that ERK2-EtsΔ138 interactions cause the phage to be released either through direct competition for similar binding sites or through non-competitive release. The non-competitive release could be due to an EtsΔ138-driven conformational change of b-ERK2 which does not allow the phage to stay bound.

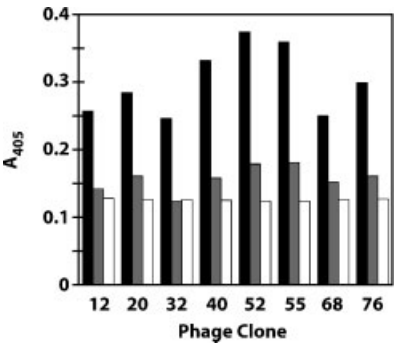


Figure 2. Phage binding to b-ERK2 decreases in the presence of a reducing agent. An ELISA was performed using $\sim 1 \times 10^8$ TU of individually purified phage clones and 500 ng of plated b-ERK2 in the presence of 0 mM (black) and 10 mM dithiothreitol (gray) during phage binding. A control was performed in the absence of b-ERK2 (white), revealing background phage binding to wells lacking DTT

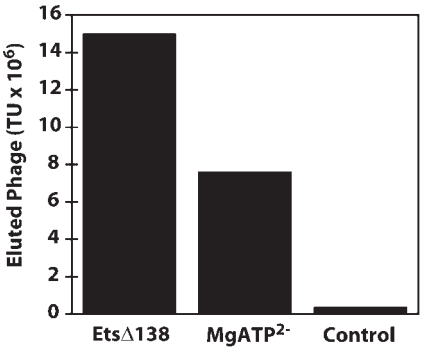


Figure 3. Elution of phage that bind b-ERK2 in round III using ERK2 substrates EtsΔ138 and ATP. Biopanning was carried out using 500 ng of plated b-ERK2 and 2×10^{11} TU of Round III input phage. Phage were allowed to bind for 1 h, washed (12×5 min) in buffer B2 containing 0.1% Tween-20 and, instead of acid elution, phage were eluted for 1 h using either 233 μM EtsΔ138 in S0 buffer (EtsΔ138), 2 mM ATP in S0 buffer (ATP) or S0 buffer (control)

A competition phage ELISA was carried out with phage selected from Round IV using several concentrations of EtsΔ138 to compete with phage binding using a limited quantity of b-ERK2. The results indicate that the presence of EtsΔ138 decreased phage binding to b-ERK2 in a concentration-dependent manner (Fig. 4). Conversely, the addition of BSA, a protein that does not bind ERK2, did not have a concentration-dependent effect (data not shown). The data indicate that a protein capable of binding ERK2 can release the bound phage or prevent them from binding since EtsΔ138 is added prior to the addition of the phage. Whether or not EtsΔ138 is competing with phage for a similar binding site or simply detaching phage due to a conformational change of the enzyme upon substrate binding cannot be elucidated from these experiments.

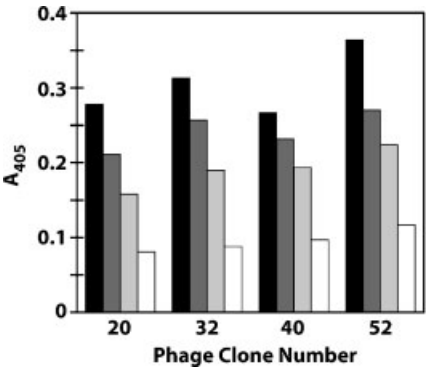


Figure 4. Transcription factor EtsΔ138 reduces phage binding b-ERK2 in a phage ELISA. An ELISA was performed using 500 ng of b-ERK2 and $\sim 1 \times 10^8$ TU of individually purified phage clones in the presence of 0 μM (black), 100 μM (dark gray) and 200 μM (light gray) EtsΔ138 added 10 min prior to phage. A control was performed in the absence of b-ERK2 and in the presence of phage (white)

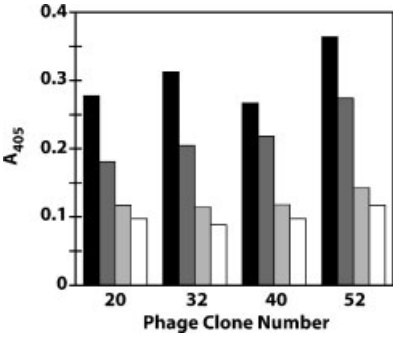


Figure 5. $MgATP^{2-}$ reduces phage binding b-ERK2 in a phage ELISA. An ELISA was performed using 500 ng of b-ERK2 and $\sim 1 \times 10^8$ TU of individually purified phage clones in the presence of 0 mM ATP and 0 mM $MgCl_2$ (black), 5 mM ATP and 10 mM $MgCl_2$ (dark gray) and 10 mM ATP and 10 mM $MgCl_2$ (light gray). A control was performed in the absence of b-ERK2 and in the presence of phage (white)

$MgATP^{2-}$ inhibits phage binding

The effects of a smaller non-protein substrate on phage binding were also studied using $MgATP^{2-}$ to elute phage during biopanning. $MgATP^{2-}$ binds ERK2 in a hydrophobic pocket and is required for protein phosphorylation. In the absence of a protein substrate, ERK2 can bind $MgATP^{2-}$ and carry out its hydrolysis to form the products ADP and inorganic phosphate. $MgATP^{2-}$ showed the ability to elute phage during Round III biopanning (Fig. 3) and inhibit phage binding in a phage ELISA in a concentration-dependent manner (Fig. 5). The mechanism by which $MgATP^{2-}$ is capable of eluting phage bound to b-ERK2 during biopanning and in a competition ELISA cannot be elucidated by these experiments but is possibly due to competitive binding or through an indirect conformational change upon binding.

Inhibition of ERK2 by cyclic peptide 20a

Since the phage displaying peptide 20 was predominantly selected in Rounds III and IV, this peptide was chemically synthesized and examined for inhibition of ERK2 as measured by the phosphorylation of the protein substrate EtsΔ138. This experiment accomplishes two goals: (i) the characterization of the peptide binding ERK2 in the absence of the phage coat proteins and (ii) an interaction study with active ERK2 in the absence of biotin to assure that the biotin moiety is not being recognized by the peptide. Unfortunately, peptide 20 was not readily soluble at neutral pH. To circumvent this problem, the peptide was synthesized with three lysines attached to the N-terminus (N-KKKIRCIRGWTKDIRTLADSCQY-C, termed peptide 20a hereafter) that increased the solubility of the peptide significantly. Peptide 20a was purified using reversed phase HPLC, oxidized to form a cyclic peptide with a disulfide bond formed between the cysteine residues, and re-purified using reversed-phase HPLC. Oxidation was confirmed by mass using ESI by revealing the loss of two protons due to cyclization.

Peptide 20a inhibited ERK2 phosphorylation of EtsΔ138 and a mode of inhibition was assigned for the substrates EtsΔ138 and $MgATP^{2-}$ (Table 3). Inhibitors are classified according to whether they affect the apparent specificity constant $(k_{cat}/K_m)^{app}$ by affecting only the apparent Henri–Michaelis–Menten constant K_m (competitive inhibition), only the apparent observed rate constant k_{cat}^{app} (uncompetitive inhibition) or both (mixed inhibition). By plotting the data in reciprocal form as $1/v$ against 1/[substrate] at various concentrations of inhibitor, one can determine the mechanism of inhibition by noting whether an inhibitor affects the slope or intercept of a plot. A competitive inhibitor increases the slope while maintaining a constant y-intercept, an uncompetitive inhibitor increases the intercept while maintaining a constant slope and a mixed inhibitor affects both the slope and intercept of such plots. A mixed inhibition pattern was seen for peptide 20a with respect to $MgATP^{2-}$ (Fig. 6) and a competitive inhibition pattern with respect to EtsΔ138 (Fig. 7).

As seen in Scheme 1, a productive complex leading to a phosphorylated product (P) is formed when ERK2 binds both $MgATP^{2-}$ (ATP) and EtsΔ138 (S). In order to bind EtsΔ138, ERK2 cannot be bound to the peptide 20a inhibitor (I) as determined by the competitive inhibition results. Since the inhibition of ERK2 by peptide 20a is mixed with respect to $MgATP^{2-}$, the enzyme can bind ATP in the presence and absence of the inhibitor.

Table 3. Inhibition patterns for the phosphorylation of EtsΔ138 by eRK2[a]

Varied substrate	Fixed substrate	Inhibitor	Mechanism	k_{obs} (s^{-1})	K_m (μM)	K_i^{app} (μM)	K_{ii}^{app} (μM)
$MgATP^{2-}$[b]	EtsΔ138[c]	Peptide[d]	Mixed[e]	15.9 ± 0.4	91.3 ± 9.5	76.7 ± 32.9	91.0 ± 11.9
EtsΔ138[f]	$MgATP^{2-}$[g]	Peptide[d]	Competitive[h]	15.3 ± 0.6	13.0 ± 2.2	20.7 ± 5.5	—

[a] Initial velocities were measured using 1 nM ERK2, 20 mM $MgCl_2$, 62.5–2000 μM ATP and 6.25–200 μM EtsΔ138, 20 mM HEPES, pH 7.3, 0.1 mM EDTA, 0.1 mM EGTA, 27 °C, and an ionic strength of 0.1 M (KCl).
[b] 62.5–2000 μM.
[c] 25 μM.
[d] 0–50 μM.
[e] Best fit of the data according to $V = (V_{max}[S])/\{K_m(1 + [I]/K_i) + [S](1 + [I]/K_{ii})\}$ for mixed inhibition using global fitting in the program Scientist.
[f] 6.25–200 μM.
[g] 270 μM.
[h] Best fit of the data according to $V = (V_{max}[S])/\{K_m(1 + [I]/K_i) + [S]\}$ for competitive inhibition using global fitting in the program Scientist.

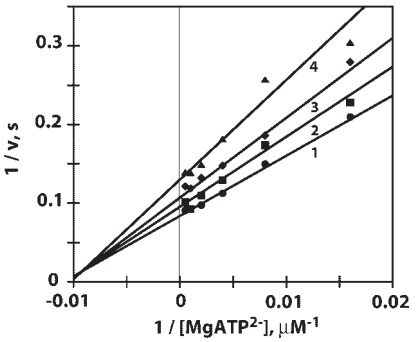


Figure 6. Cyclic peptide 20a exhibits a mixed inhibition mechanism of ERK2 with respect to $MgATP^{2-}$ Initial velocities were measured using 2 nM ERK2, 20 mM $MgCl_2$ and 25 μM EtsΔ138 in the presence of several fixed concentrations of peptide 20a (1, 0 μM; 2, 12.5 μM; 3, 25 μM; and 4, 50 μM), various concentrations of ATP (62.5--2000 μM), 20 mM HEPES, pH 7.3, 0.1 mM EDTA, 0.1 mM EGTA, 27 °C, and an ionic strength of 0.1 M (KCl). The data were globally fitted to the equation $V = (V_{max}[S])/\{K_m(1 + [I]/K_i) + [S](1 + [I]/K_{ii})\}$ using the program Scientist

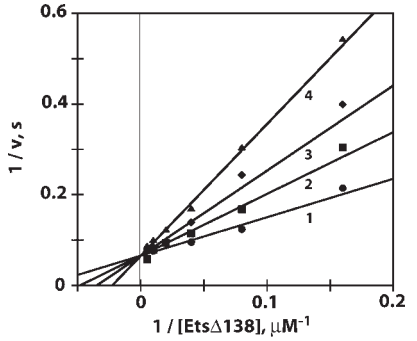
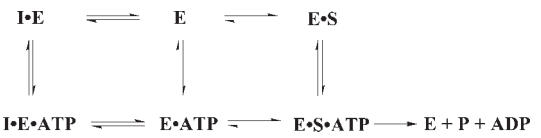


Figure 7. Cyclic peptide 20a exhibits a competitive inhibition mechanism of ERK2 with respect to EtsΔ138. Initial velocities were measured using 1 nM ERK2, 20 mM $MgCl_2$ and 270 μM ATP in the presence of several fixed concentrations of peptide 20a (1, 0 μM; 2, 12.5 μM; 3, 25 μM; and 4, 50 μM), and various concentrations of EtsΔ138 (6.25--200 μM), 20 mM HEPES, pH 7.3, 0.1 mM EDTA, 0.1 mM EGTA, 27 °C, and an ionic strength of 0.1 M (KCl). The data were globally fitted to the equation $V = (V_{max}[S])/\{K_m(1 + [I]/K_i) + [S]\})$ using the program Scientist



Scheme 1

Therefore, peptide 20a binds a region of ERK2 that is distinct from the $MgATP^{2-}$ binding site.

The kinetic inhibition data are in accord with the experimental evidence that EtsΔ138 competed with phage 20 in a dose-dependent manner for binding to ERK2 in a competition ELISA (Fig. 4). The $K^{app}_{i(Ets\Delta138)}$, a measure of the affinity of the peptide for the enzyme, has a similar affinity to the K_m of the protein substrate EtsΔ138,[24] indicating that they not only compete for the same binding site but also bind with similar affinities (Table 3), despite their difference in size. While it remains to be determined how peptide 20a competes with EtsΔ138 for ERK2 binding, it is certainly conceivable that it binds a site utilized by EtsΔ138, which lies outside the active site. This is consistent with the observation that the peptide is not a competitive inhibitor for $MgATP^{2-}$.

Inhibition is not specific to ERK2

A related member of the MAPK family, p38 MAPKα, was also inhibited by peptide 20a. In this case, the kinetic data of inhibition were best fitted to a competitive inhibition mechanism with respect to the protein substrate GST-ATF2-(1–115) ($k_{cat} = 0.7 \pm 0.1\ s^{-1}$, $K_m = 3.6 \pm 0.9$ μM, $K_i = 5.6 \pm 1.4$ μM) (data not shown). These results indicate that peptide 20a, although selected for ERK2 binding, is not specific for ERK2 and may recognize a motif shared among this family of kinases such as a docking groove. Indeed, both MAPK family members, 46% identical, have a number of regions of similarity that the peptide may be recognizing. One region could be the semi-conserved active site region responsible for peptide substrate recognition and $MgATP^{2-}$-binding. However, peptide 20a could bind ERK2 in the presence and absence of $MgATP^{2-}$, indicating that the peptide may not be targeting the active site. Both MAPKs share a similar phosphorylation loop containing a phosphorylated threonine and tyrosine residue required for the activation of each enzyme. Basic residues on peptide 20a could bind this region through recognition of negatively charged phosphate groups, thereby inactivating the enzyme. Another region of similarity between p38 MAPKα and ERK2 is the common docking domain composed of acidic residues responsible for binding substrates, activating kinases and phosphatases.[15] Several basic residues in peptide 20a could mediate the binding to this acidic region through electrostatic interactions. However, it has not yet been determined whether EtsΔ138 and GST-ATF2-(1–115) utilize the common docking domain on ERK2 and p38 MAPKα. It is also plausible that the peptide recognizes a novel hot spot, conserved amongst ERK2 and p38 MAPKα, mediating protein substrate docking.

CONCLUSIONS

We have shown that phage display can be used to select peptides that bind the active form of ERK2. One of the peptides selected, peptide 20a, was chemically synthesized and shown to competitively inhibit the ability of ERK2 to phosphorylate a model transcription factor substrate, EtsΔ138. However, the selected peptide also inhibited a closely related MAPK family member, p38

MAPKα. These results suggest that there may be a hot spot on ERK2 and p38 MAPKα that has evolved to bind ligands and serves as a superficial target for phage display. In nature, this same region on ERK2 may act to entice other ligands within the cell to bind.

Acknowledgements

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