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Proximity-Induced Catalysis by the Protein Kinase ERK2

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Protein phosphorylation regulates most aspects of life and is controlled by two families of enzymes: the protein kinases, which add phosphates to proteins, and the protein phosphatases, which remove them. In humans, approximately 500 protein kinases are responsible for the phosphorylation of some 10 000 cellular proteins.¹ Currently, it is not possible to predict with high accuracy which cellular protein will be a substrate for a particular protein kinase. The problem is complex because the structures of many of the kinases and substrates are unknown and often multiple sites in a protein are phosphorylated, frequently by more than one protein kinase. A strategy many protein kinases have adopted, to achieve their required specificity, is to focus on the recognition of a consensus phosphorylation motif, a linear signature sequence, typically of 4–8 amino acids, that encompasses a target residue of a protein.² The consensus sequence is bound, in an extended configuration, within a groove of a protein kinase in a manner that serves to position the sequence in the appropriate position for phosphoryl transfer to occur.³ In most cases, the role played by the protein structure in modulating the specificity is poorly understood.

The mitogen-activated protein kinases, a subfamily of protein kinases implicated in numerous diseases, adopt a focus different from those of most protein kinases, because their two amino acid consensus motif, S/T–P,⁴ is too common to support high specificity on its own. Not surprisingly, evidence has accumulated to support the notion that MAPKs employ an expansive mechanism of substrate recognition. In this process, instead of binding one contiguous sequence, recognition of the S/T–P motif within the active site is facilitated by protein–protein interactions that typically occur over a fairly large surface area outside the active site.^{5,6}

The transcription factor, Ets-1 (Figure 1), is one of many important and structurally diverse substrates of the MAP kinase ERK2, whose phosphorylation by ERK2 promotes transcription at rev responsive elements.⁸ ERK2 recognizes a domain in Ets-1 called the pointed (*pnt*) domain, which is a compact, five-helix bundle immediately C-terminal to an unstructured N-terminal tail that contains the sole ERK2 phosphorylation site, Thr-38 (Figure 1).⁹ There is little mechanistic information relating MAPK–protein interactions to the catalytic process. In this communication, we investigate the functional relationship between the *pnt* domain of Ets-1 and the distal TP consensus motif.¹⁰ We provide evidence for a mechanism of *proximity-mediated* catalysis, where substrate turnover proceeds through

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Supporting Information Available: Procedures for kinase and fluorescence polarization assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

the discrete formation of a docked complex, **I**₁, that is extensively stabilized by interactions involving the *pnt* domain and a region near the α_d and α_f helices and loop L13 of ERK2 (Figure 1, Scheme 1). This complex is catalytically *effective* compared to a complex with a TP-containing peptide that lacks a *pnt* domain because the *pnt* domain promotes the binding of the TP motif in the active site.

To examine the mechanistic basis of ERK2 substrate specificity, we used the substrate Ets 138, a 138-amino acid protein containing both the unstructured N-terminal tail and the pointed domain (Figure 1), and found it to be phosphorylated by ERK2 with a high specificity of k_{cat}/K_m $10^6 \text{ M}^{-1} \text{ s}^{-1}$.

To examine the role of the *pnt* domain, we first tested the ability of the F120A mutant of Ets 138 to bind ERK2. F120 lies in loop L4, distal to the TP motif. We confirmed earlier data that its mutation to alanine compromises phosphorylation by ERK2; k_{cat}/K_m for the mutant is 20-fold smaller than the wild-type enzyme, while k_{cat} remains constant (Figure 2B).⁹ Then, using a fluorescence anisotropy assay, we demonstrated that F120A binds ERK2 10-fold less tightly than the wild-type protein (Figure 2A). By definition, k_{cat} reports the rate-limiting steps that occur after substrate binding. For the phosphorylation of Ets 138 there are two rate-limiting steps: phosphorylation of Thr-38 within the ternary complex (109 s^{-1}) and the release of products (56 s^{-1}).¹² The k_{cat} data suggest that the effect of the F120A mutation is exhibited only in the initial binding step, K_1 , and that once bound, the mutant protein behaves exactly like the wild-type protein. Thus, the decrease in the specificity constant k_{cat}/K_m can be attributed to a decrease in the affinity of ERK2 for the distal *pnt* domain, K_1 , and not to a kinetic step following binding. That is, the F120A mutation does not appear to influence reaction flux through steps 2, 3, or 4 of Scheme 1.

To examine the role of the TP motif, P39 was substituted for both charged and hydrophobic residues to perturb binding of the TP motif within the active site. Given that virtually every known site of proteins phosphorylated by ERK2 is followed by a proline, it was anticipated that the TP motif of Ets 138 would contribute significantly toward substrate recognition. Therefore, we examined the ability of ERK2 to bind a number of P39X mutants (X = D, R, V, G, or A). Surprisingly, none of these substitutions significantly affected the ability of ERK2 to bind Ets 138, despite differences in both charge and size. In addition, even the double mutant T38A/P39A bound with a similar affinity to the wild-type protein (Figure 2A). These data show that the TP motif does not contribute to specificity through stabilizing the ERK2•Ets 138 binary complex. [This is supported by the observation that deletion of residues 24–50 (numbered as in Ets-1) from Ets 24–138 decreases binding by less than 2-fold (data not shown)]. The minor variations seen in the binding of the proteins can be attributed to either solvation effects or minor perturbations on the protein dynamics. The simplest explanation for the observed insensitivity of the dissociation constant to the mutations is that the active site of ERK2 is not occupied by the TP motif in the binary complex.

As we found no evidence for TP motif-induced stabilization of the binary complex, we considered its role in stabilizing the ternary complex. Therefore, we examined the ability of both Ets 138 and the Ets 138 T38A/P39A mutant to bind ERK2 in the presence and

absence of a saturating concentration (2 mM) of the ATP analogue AMP-PNP, which is a competitive inhibitor of ATP with a K_i of $\sim 25 \mu\text{M}$ (Callaway, K; unpublished observations). A productive interaction between MgAMP-PNP and the TP motif would cause an increase in the affinity of wild-type Ets 138, but not the mutant. Surprisingly, the addition of AMP-PNP, in the presence of 10 mM Mg^{2+} , had no effect on the dissociation constant of either Ets 138 or of the mutant (data not shown), suggesting that the consensus motif makes little contribution to the stability of the ternary complex. [The variation in the K_m of each mutant protein (data not shown) was consistent with the conclusions drawn from the binding data.]

Taken together, the evidence supports the mechanism in Scheme 1, which for simplicity, assumes that MgATP is bound to ERK2. According to this mechanism, ERK2 binds the *pnt* domain of Ets 138, K_1 , to form complex **I**₁. We propose that **I**₁ is on the reaction pathway for phosphoryl transfer to T38 and that it undergoes a conformational rearrangement, K_2 (where $K_2 < 1$) to form the activated ternary complex **I**₂. **I**₁ is catalytically incompetent because the TP motif does not occupy the active site. In contrast, **I**₂ is catalytically competent because it is characterized by the binding of the TP motif within the active site in such a manner that T38 can hydrogen bond to the catalytic base, D147, priming it for phosphoryl transfer, k_3 . Mutagenesis of the TP motif will destabilize **I**₂ but not the ERK2•Ets 138 complex, because the contribution to its stability from **I**₂ is minimal.

To lend further support to the model we tested the ability of ERK2 to phosphorylate the P39 mutant proteins. As predicted, the mutant proteins showed differential abilities to serve as substrates (Figure 2B). While the charged mutants P39D and P39R were not phosphorylated (despite binding), P39A, P39V, and P39G were phosphorylated with appreciable values of k_{cat} . The simplest explanation for the effects of the P39X mutations on k_{cat} is that they destabilize the equilibrium K_2 and increase the transition-state energy for phosphoryl transfer, k_3 .

This study suggests how a promiscuous MAPK with a large number of structurally diverse substrates (perhaps as many as 50) might use interactions outside the active site to promote catalytic events within it. Because TP motifs on peptides do not generally bind with enough affinity to the active site of ERK2, for catalysis to be effective, ERK2 utilizes a docking site on a protein to effectively increase the concentration of a peptide sequence near the active site. In the case of Ets-1, the *pnt* domain provides a docking site to which ERK2 binds. While further work is required to understand the mechanism in detail, the analysis of the F120A mutant supports the notion that the ERK2-*pnt* interaction provides a uniform binding interaction, which is used to stabilize subsequent intermediate and transition states on the reaction pathway by an equal amount. We are currently testing whether this mechanism is generally applicable to ERK2 as well as other proline-directed protein kinases, such as the p38 MAPKs, the JNKs and the cyclin-dependent protein kinases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Functional domains of murine Ets-1. Structure of Ets 138 (PDB 1BQV) and structure of ERK2 (PDB 2ERK) showing the conserved active site base, Asp-147 (red), and the *pnt* docking site (yellow star), comprising the α_d and α_f helices and loop L13.⁷

Figure 2. Effect of mutations in both the *pnt* domain and the TP motif of Ets 138 on (A) K_d for Ets 138•ERK2 dissociation and (B) k_{cat} .

Scheme 1.
Reaction Mechanism of ERK2