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A single amino-acid change in ERK1/2 makes the enzyme susceptible to PP1 derivatives

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Abstract

We generated extracellular signal-regulated kinase 1/2 (ERK1/2) mutants by introducing a single amino-acid substitution in subdomain V of the catalytic domain and then examined the susceptibility of these mutants to PP1 derivatives originally designed as Src inhibitors. Substituting smaller amino acids (alanine [Ala (A)] or glycine [Gly (G)]) for glutamine [Gln (Q)] in subdomain V drastically increased the susceptibility of ERK1/2 to 1-naphthyl PP1 (1NA-PP1). Wild-type ERK1/2 was resistant to 1NA-PP1 inhibition. ERK1(Q122A) and ERK2(Q103A) were inhibited by 1NA-PP1 at IC_{50} values of 1.7 ± 0.13 and 2.1 ± 0.18 μM, respectively. ERK1(Q122G) and ERK2(Q103G) were inhibited by 1NA-PP1 with IC_{50} values of 3.6 ± 0.26 and 18 ± 2.2 μM, respectively. Other derivatives of PP1 (1-naphthylmethyl PP1 and 2-naphthylmethyl PP1) did not significantly inhibit ERK1/2 and its various mutants. In addition, these ERK1/2 mutants were activated by TPA when they were expressed in mammalian cells. These results suggest that the Gln residue of subdomain V is important in determining the susceptibility of ERK1/2 to 1NA-PP1 without significant changes in their enzymatic characteristics.

Keywords: ERK; Mutagenesis; Src inhibitor

Protein kinases play essential roles in many cellular functions. Assigning precise functional roles to many kinases, however, is complicated by the possible functional redundancy in their ATP-binding sites that are major targets of synthetic inhibitors [1]. Due to high sequence homology, kinases have several conserved subdomains in their catalytic domains, one of which, subdomain V, represents the catalytic site [1]. A recent chemical-genetic approach showed that a single amino-acid mutation introduced in subdomain V makes certain kinases, such as tyrosine kinases (v-Src, c-Fyn, and c-Abl) and Ser/Thr kinases (CaMKII, CDK2, and Cdc28), very susceptible to PP1 derivatives, without significantly affecting their catalytic activities [2].

ERK1 and ERK2 have a conserved Gln residue located at similar positions in subdomain V (Fig. 1). This prompted us to examine whether mutation of the Gln residue in ERK1/2 also causes susceptibility to PP1 derivatives originally designed as Src inhibitors [3]. Our kinase assay results show that substitution of Gln with a smaller amino acid residue makes ERK1/2 extremely susceptible to a PP1 derivative. Wild-type ERK1/2, however, was unaffected by the inhibitor. Our results may motivate the search for and development of specific inhibitors of ERK1/2, a kinase that plays an essential role in a variety of cell functions.

Materials and methods

PP1 analogs. PP1 analogs were synthesized as described previously [3].

Generation of ERK1 and ERK2 mutants. We obtained ERK1 cDNA by screening a mouse NIH 3T3-L1 cDNA library constructed in lambda

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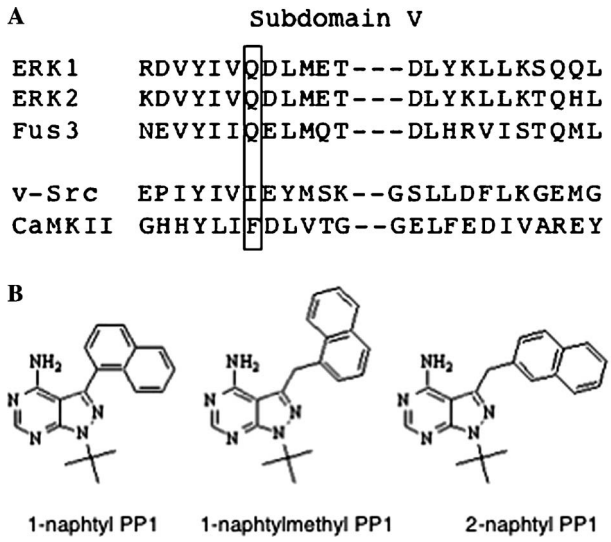

Fig. 1. Comparison of subdomain V of different kinases and of structures of PP1 derivatives. (A) Mutation of the indicated amino acids (boxed residues) to Gly or Ala makes these kinases extremely susceptible to PP1 derivatives. (B) Structures of PP1 derivatives that are Src inhibitors.

ZAPII (Stratagene). The *Eco*RI fragment of the cDNA (1.8 kbp) was blunt-ended with a Klenow fragment and was cloned into the *Stu*I site of pMAL-c (New England Biolabs). This *Eco*RI fragment of ERK1 lacks the first 6 amino acid residues of mouse ERK1. A full-length mouse ERK2-expressing plasmid was generated via PCR cloning from the mouse brain lambda gt10 cDNA library (Clontech) with the following primers that contain *Eco*RI and *Xba*I sites (underlined): 5′-TAGAATTCAA GCGTCGAACCGAA-3′ and 5′-ATACAGATCTTTAATTGCTCTA GAGC-3′.

Amplification was carried out at 94 °C for 30 s, 45 °C for 30 s, and 65 °C for 30 s. The PCR product (1.2 kbp) was purified and digested with *Eco*RI and *Xba*I and cloned into pGEX4T (pGEX4T-ERK2) for expression of ERK2 as a GST fusion protein (GST-ERK2) in *Escherichia coli*. To confirm the ERK1/2 sequences, both strands of the plasmids were sequenced with ABI310 (Applied Biosystems) and BigDye terminator cycle sequencing kit (Applied Biosystems).

We carried out double-stranded, site-directed mutagenesis on pMAL-ERK1 and pGEX4T-ERK2 using Quikchange (Stratagene) with the following primers (mutations are underlined): ERK1(Q122G), 5′-ATATA TAGTAGGGGACCTCATGGAGA-3′ and 5′-TCTCCATGAGGTC CCCTACTATATAT-3′; ERK1(Q122A), 5′-ATATATAGTAGCGGAC CTCATGGAGA-3′ and 5′-TCTCCATGAGGTCCGCTACTATATAT-3′; ERK2(Q103G) 5′-GTATATATAGTAGGGGACCTCATGG-3′ and 5′-CCATGAGGTCCCCTACTATATATAC-3′; ERK2(Q103A), 5′-GTA TATATAGTAGCGGACCTCATGG-3′ and 5′-CCATGAGGTCCGC TACTATATATAC-3′. These mutations were confirmed by double-strand sequencing of the constructs, as mentioned above.

Bacteria (BL21-codonPlus-RIL, Stratagene) transformed with pMAL-ERK1 or pGEX4T-ERK2 were grown in CircleGrow (BIO101) at 37 °C until the A_{600} reached 1.0. The expression of recombinant MBP-ERK1 and GST-ERK2 was induced by adding 1 mM IPTG and incubating for 12 h at 37 °C. For pMAL-ERK1(Q122G), transformed bacteria were grown at 20 °C; induction was also carried out at 20 °C. MBP-ERK1 mutants were purified using amylose-resin (New England Biolabs) and GST-ERK2 mutants were purified using glutathione–Sepharose (Amersham Pharmacia) followed by ion-exchange chromatography using ResourceQ (Amersham Pharmacia). We determined protein concentration according to the method of Bradford [4], using BSA as a standard ($E^{1\%}_{280} = 4.56$). SDS–PAGE was carried out according to the method of Laemmli [5]. The proteins were stained with Coomassie brilliant blue.

Kinase assay for measuring ERK1/2 activity. PP1 compounds were dissolved in DMSO. DMSO was used as a control. The final concentration of DMSO in the reaction mixture was kept constant at 1%. This concentration of DMSO had no effect on the activities of ERK1/2 or their mutants (data not shown). The activities of recombinant MBP-ERK1 and GST-ERK2 were measured at 30 °C for 30 min in the reaction mixture (50 μl), which contained 50 μM myelin basic protein, 50 μM [γ-^{32}P]ATP, 2 mM $MgCl_2$, 25 mM Hepes–NaOH (pH 7.6), and 25 mM β-glycerophosphate in the presence of various concentrations of PP1 derivatives. The reaction mixture (40 μl) was spotted onto P81 membranes (Whatman), which were washed four times in 75 mM H_3PO_4. The membranes were dried, placed in liquid scintillation cocktail, and subjected to liquid scintillation counting.

Activation of ERK1/2 mutants expressed in CHO cells by TPA stimulation. ERK1(wild type, Q/A, Q/G), ERK2 (wild-type, Q/A, Q/G) were inserted into pCMV-Flag-2 (Sigma) and kinase-deficient mutant (ERK2(K52A)) was inserted into pCMV-HA (Clonetech). The plasmid containing wild-type and mutant ERK1/2 was transfected with CHO cells using FuGENE6 (Roche) for the transient expression of ERK1/2 mutants. The cells were serum-starved with 0.2% fetal bovine serum for 24 h. Then, the cells were treated with 0.2 μM TPA for 30 min. Wild type and mutant ERK1/2 were immunoprecipitated from cell lysates by using antibody against FLAG or HA tag. The activation of ERK was measured by using MAP kinase assay kit using myelin basic protein as a substrate (Upstate Cell Signaling Solutions) according to the manufacturer's instruction. To determine the loading of the ERKs in each lane, immunoblots were carried out using anti-tag antibody (anti-FLAG or anti-HA) on the duplicate blot obtained in the ERK assay mentioned above.

Results and discussion

The highly conserved catalytic domains of Ser/Thr kinases and tyrosine kinases are divided into 12 subdomains [1]. Subdomains I–IV form the small lobe that is mainly involved in binding and orienting the nucleotide, whereas subdomains VI–XII form the large lobe that is primarily responsible for binding the peptide substrate and transferring phosphate to the substrate. Subdomain V spans the two lobes and forms the catalytic site. Subdomain V is well conserved among protein kinases including tyrosine kinases, Ser/Thr kinases, and dual-specificity kinases [1] (Fig. 1). Recently, it was discovered that a functionally conserved residue in the V subdomain of the kinase active site (I338 in v-Src; Fig. 1) could be mutated to an Ala or a Gly to create a new pocket in the active site [6–8]. Mutation of this residue to an Ala or a Gly effectively allows efficient catalysis with ATP analogs [6,9] and inhibition by orthogonal PP1-derived inhibitors [2]. In the present study, we have extended this approach to ERK1/2 kinases.

ERK1/2 plays a variety of physiological roles in cell differentiation, development, and neuronal plasticity [10]. Because MEK1/2 is thought to be the primary enzyme that phosphorylates and activates ERK1/2 in vivo [11], MEK1/2 inhibitors, such as PD98059 and U0126, are widely used to decipher the role of ERK1/2 in biological phenomena. Even though MEK inhibitors have proved to be essential tools in examining the roles of MEK–ERK pathways in signaling cascades, due to the lack of specific inhibitors for ERK1/2, it has been difficult to assess the role of basal ERK1/2 activities or the MEK-independent activation of ERK1/2 in cellular processes [12]. This prompted us to create mutant ERK1/2-inhibitor pairs to examine the function

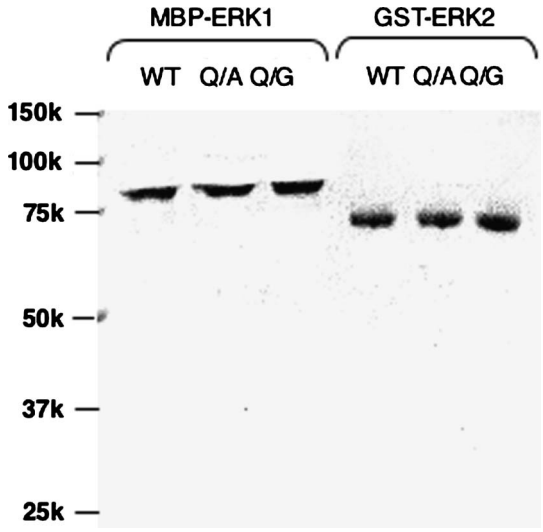

Fig. 2. Purification of MBP-ERK1 and GST-ERK2. MBP-ERK1 and GST-ERK2 were expressed in *E. coli* and purified by affinity chromatography and ion-exchange chromatography (see Materials and methods). The obtained proteins were subjected to SDS–PAGE, and the gel was stained with Coomassie brilliant blue. Each lane contains 0.5 μg protein.

of ERK1/2. We assumed that mutations introduced into subdomain V of ERK1/2 (Fig. 1) render ERK1/2 susceptible to derivatives of PP1 [3].

We substituted either Ala (A) or Gly (G) for Gln (Q) in subdomain V of mouse ERK1 and ERK2 using site-directed mutagenesis (Fig. 1). The introduction of mutations was confirmed by double-strand DNA sequencing of the plasmids. The recombinant MBP-ERK1 and GST-ERK2 fusion proteins were expressed in *E. coli* and purified by affinity chromatography (amylose–resin and glutathione–Sepharose) and ion-exchange chromatography (Fig. 2). For some unknown reason, the yield of MBP-ERK1(Q122G) was significantly lower than those of MBP-ERK1(Q122A) and MBP-ERK1(WT) when the expression was carried out at 37 °C. The yield of MBP-ERK1(Q122G) was satisfactory, however, when the expression was carried out at 20 °C. SDS–PAGE of the recombinant proteins revealed that they were greater than 90% pure after purification via affinity and ion-exchange chromatography (Fig. 2). Except for the specific activity of MBP-ERK1(Q122G), which was one-fourth of that of wild-type MBP-ERK1, there were no significant differences between the specific activities of wild-type ERK and mutant ERKs when myelin basic protein was used as a substrate (0.6–0.8 μmolPi/min/mg protein) (Table 1). One possible reason for the diminished specific activity of MBP-ERK1(Q122G) is that expressing the enzyme at 20 °C may have decreased autophosphorylation.

We examined the susceptibility of ERK1/2 mutants to PP1 derivatives by measuring their activities in the presence of various concentrations of three different PP1 derivatives and comparing their activities to those of wild-type ERK1/2. Two PP1 derivatives, 1-naphthylmethyl-PP1 and 2-naphthylmethyl-PP1, failed to inhibit wild-type ERK1/2 and ERK1/2 mutants even at concentrations of 1000 μM. We were unable to test the effects of PP1 concentrations greater than 1000 μM using our kinase assay, and thus could not obtain precise IC_{50} values for these two compounds. On the other hand, 1-naphthyl-PP1 (1NA-PP1) strongly inhibited ERK1(Q122A) and ERK1 (Q103G) at IC_{50} values (±SD) of 1.7 ± 0.13 and 3.6 ± 0.26 μM, respectively. However, 1NA-PP1 failed to inhibit wild-type ERK1/2 (Fig. 3, Table 1), even at a concentration of 1000 μM. Similarly, 1NA-PP1 inhibited ERK2(Q122A) and ERK2 (Q103G) at IC_{50} values of 2.1 ± 0.18 and 18 ± 2.2 μM, respectively, but failed to inhibit wild-type ERK2 (Fig. 3, Table 1). Interestingly, even though 1NP-PP1 at concentrations as high as 1 mM failed to inhibit wild-type ERK1/2, the IC_{50} values we measured for ERK1/2 Q/A or Q/G mutants were significantly higher than those measured for mutants of Src and other kinases [2]. Furthermore, PP1 derivatives having IC_{50} values in the nanomolar range inhibited the mutants of Src and other kinases, even though the corresponding wild-type kinases were essentially insensitive to the inhibitors at this concentration [2,3]. Our results suggest that structures other than those affected by the mutated site may be required to increase the susceptibility of ERK1/2 to PP1 derivatives. Further analysis is required to identify the structural differences between subdomain V of ERK1/2 and those of other kinases, since such differences may be responsible for the variable inhibition of different kinases by PP1 derivatives.

Fig. 1 shows the aligned amino acid sequences of subdomain V of kinases analyzed thus far for their susceptibility to PP1 derivatives. For ERK1/2, the Gln residue, which we

Table 1
Inhibition of ERK1/2 and ERK1/2 mutants by PP1 derivatives[a]

Enzyme	Relative activity (%)	IC_{50} (μM) of PP1 derivatives[b]		
		1-Naphthyl	1-Naphthylmethyl	2-Naphthylmethyl
MBP-ERK1 (WT)	(100)	>1000	>1000	>1000
MBP-ERK1 (Q/A)	88	1.7 ± 0.13	>1000	>1000
MBP-ERK1 (Q/G)	23	3.6 ± 0.26	>1000	>1000
GST-ERK2 (WT)	(100)	>1000	>1000	>1000
GST-ERK2 (Q/A)	78	2.1 ± 0.18	>1000	>1000
GST-ERK2 (Q/G)	83	18.0 ± 2.20	>1000	>1000

[a] The activities of ERK1/2 and ERK1/2 mutants were measured by using myelin basic protein as a substrate (see Materials and methods).
[b] IC_{50} values (±SD) represent the average of values obtained from 3 to 4 separate experiments.

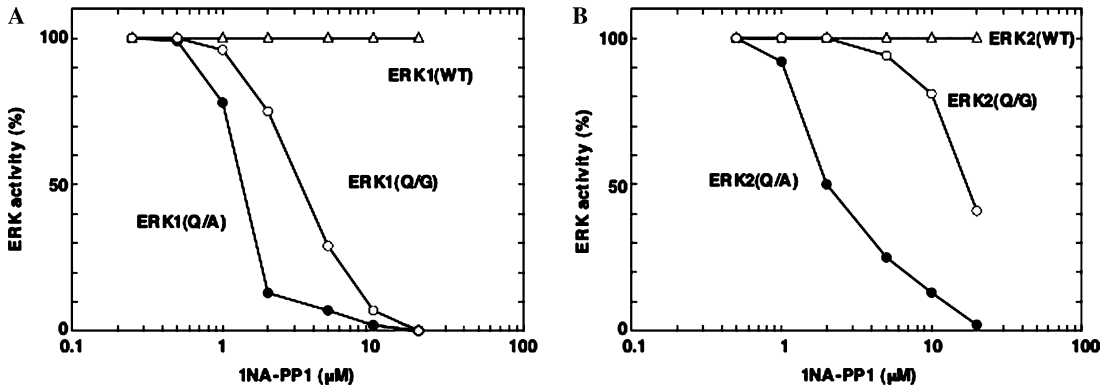

Fig. 3. Inhibition of ERK1/2 and their mutants by 1-naphthyl PP1. (A) Inhibition of ERK1 and ERK1 mutants by 1NP-PP1. (B) Inhibition of ERK2 and ERK2 mutants by 1NP-PP1. ERK1/2 activities were determined by measuring ^{32}Pi incorporation into myelin basic protein (see Materials and methods). Each point represents the average of triplicate experiments.

substituted with either Ala or Gly, aligned with amino acids having bulky side chains, such as Ile (v-Src), Thr (c-Fyn), Phe (CaMKII), and Thr (CDK2). Substituting these bulky amino acid residues with residues having small side chains (e.g., Gly and Ala) rendered these kinases susceptible to inhibition by PP1 derivatives [2]. Taken together, these findings indicate that the small side chains of Gly or Ala in the mutated kinases allowed the bulky inhibitors to bind the catalytic site formed by subdomain V. Ala mutants of ERK1/2 were more susceptible to 1NA-PP1-mediated inhibition than were Gly mutants, suggesting that PP1 susceptibility may be determined not only by the size of the amino acid side chains but also by the interaction of the side chains with the functional groups of the inhibitors. As seen with the kinases assessed by Bishop et al. [2], PP1 derivatives were highly selective for inhibiting ERK1/2(Q/G) and ERK1/2(Q/A) when compared to wild-type ERK1/2 (Fig. 3, Table 1). Although we were unable to determine the precise IC_{50} values of wild-type ERK1/2 in the present study, from our findings, we conclude that the 1NA-PP1 selectivity for the ERK mutants is at least 500-fold greater than its selectivity for wild-type ERK (1000/1.7-fold for ERK1(WT)/ERK1(Q/A) and 1000/2.1-fold for ERK2(WT)/ERK2(Q/A)). The selectivity of PP1 derivatives for mutant kinases versus wild-type kinases might be similar to that for Src kinases [2].

The activation of ERK1/2 mutants was determined by introducing ERK1/2 mutants in CHO cells (Fig. 4) to examine the basic characteristics of ERK1/2. The ERK1 (wild type, Q/A, and Q/G), ERK2 (wild type, Q/A, and Q/G), and kinase-deficient mutant ERK2 (K/A) were transfected with the plasmid containing ERK1/2 mutants. The CHO cells were stimulated with TPA for the activation of ERK1/2 after serum starvation. The 30-min treatment of CHO cells expressing ERK1/2 mutants led to the activation of all of ERK1/2 except for ERK2(K/A). The results suggest that ERK1(Q/A, Q/G), ERK2(Q/A, Q/G) still conferred the activation to the stimuli such as TPA though they have mutations in subdomain V.

When determining the physiological functions of a particular kinase, using membrane permeable inhibitors such as PP1 derivatives has several advantages over deleting kinase genes by means of gene targeting techniques. First, kinase inhibitors affect target kinases quickly and reversibly. Second, because mutated kinases usually retain their activities (Table 1), the compensation for the activity of the deleted kinase during the development can be avoided. Most inhibitors bind to the ATP-binding site, which is conserved among kinases [1]. Thus, one drawback with using standard kinase inhibitors is that their low specificity for individual kinases makes it difficult to determine whether the inhibitory effects observed are solely due to the inhibition of the target kinase.

Recently, ERK1 knockout mice have been developed [13–16]. The unusual phenotype of these mice suggests that ERK1 plays an important role in a variety of tissues. Although the gene knockout technique is one of the most powerful tools available today for clarifying the physiological role of enzymes, issues arise regarding the validity of using null knockout mice to determine the function of tar-

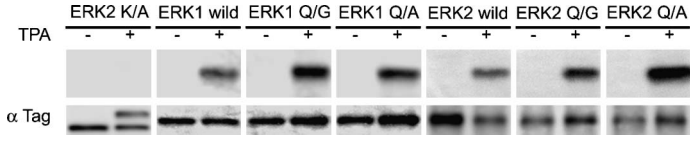

Fig. 4. Activation of ERK1/2 and their mutants expressed in CHO cells. The wild type and mutants of ERK1/2 were transiently expressed in CHO cells. The serum-starved cells were treated with TPA for 30 min and then the activation of ERK1/2 mutants was examined using myelin basic protein as a substrate. The phosphorylation of myelin basic protein was examined by immunoblot analyses using antibody specific for phosphorylated myelin basic protein. Each experiment was repeated 2–3 times and representative data are shown in the figure. Upper panels show the immunoblot using anti-phosphorylated myelin basic proteins and lower panels the expression and loading of ERK1/2 mutants examined by anti-FLAG or anti-HA antibody.

geted enzymes during development, since null knockout mice lack the targeted enzymes from the beginning of development. The development of the inhibitor-kinase pair technique by Bishop et al. [2] made it possible for us to generate mice that express inhibitor-sensitive kinase but not endogenous kinase. These types of mice will allow us to examine the physiological role of a kinase by applying kinase-specific inhibitors that reversibly turn off the kinase's activity in any tissue or cell type at any time.

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