Characterization of Phosphorylation Sites from the Activation Loop of the Mitogen-Activated Protein Kinase ERK1

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Abstract

Reversible protein phosphorylation catalyzed by protein kinases is universally employed by eukaryotes to regulate enzyme activity, protein-protein interactions, subcelluar localization and protein turnover. The catalytic domains of most eukaryotic protein kinases are conserved in their primary sequences, particularly in up to 12 functionally important subdomains. The activation loop, a variable region between subdomains VII and VIII, is usually phosphorylated at multiple sites to facilitate maximal activation of typical protein kinases. From analysis of the distribution and evolutionary conservation of phosphosites in 496 human protein kinase catalytic domains, two phosphosites within the activation loop (T154 and Y157 from the alignment of all human protein kinase domains) were identified as the most conserved sites in almost half of the human protein-serine/threonine kinases. Mitogen-activated protein kinases (MAPKs) play fundamental roles in the control of cell growth and proliferation from yeast to humans, and they have served as a paradigm for the study of protein kinase regulation. In all of the MAPK members, there are three neighboring threonine and tyrosine phosphosites in addition to those within the well documented "TXY" activation motives that are targeted by MAPK kinases (MEKs). The functions of these flanking phosphosites are unclear. Based on their high evolutionary conservation rates and the general activatory effect of phosphosites within kinase activation loop, we hypothesized that these three flanking sites could be critical for full activation of MAPKs. Each of these phosphosites were mutated individually and in combination, and assayed for their kinase activities. We also used active MEK1 to phosphorylate a kinase-dead (KD) mutant of ERK1, in order to explore the possibility of autophosphorylation on the flanking sites. From our results, we propose that the T207 and Y210 phosphosites of ERK1, which correspond to the most conserved T154 and Y157 phosphosites, are autophosphorylated after the phosphorylation of "TEY" by MEK1, and this is required for full activation of ERK1. Our findings contribute to an improved knowledge of MAPK regulation as well as protein kinases in general.

Introduction

Protein kinases mediate most intracellular signal transduction via the reversible phosphorylation on serine, threonine, and tyrosine residues of specific protein substrates. Such phosphorylation provides an efficient means to regulate almost most physiological activities including metabolism, transcription, DNA repair, cell growth, division, and apoptosis. Eukaryotic protein kinases comprise a broadly expanded family of proteins that feature a conserved catalytic domain. Within the catalytic domain, twelve extremely conserved subdomains involved in ATP binding and the phosphotransfer reaction have been well characterized. Our group has been investigating the distribution and evolutionary conservation of phosphosites in 496 human protein kinase catalytic domains. All of the experimentally confirmed phosphorylation sites were mapped following an alignment of the amino acid sequences of all of these catalytic domains (Figure 1). About 75% of the known activating phosphosites are located within the activation loop, a variable region between subdomains VII (DFG) and VII (APE). From our further evolutionary analysis, phosphosites at positions 154 and 157 of the alignment were identified as the most conserved sites in many human protein-serine/threonine kinases, including MAPKs, CDKs, PKCs and PKB/AKTs (Table 1).

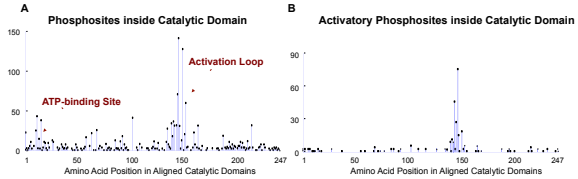

Figure 1. Distribution of all the confirmed phosphosites (A, total = 1942) and activation sites (B, total = 303) in the alignment of 496 human protein kinase catalytic domains.

	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161
CDK1	P	I	R	T	Y	T	H	E	V	V	T	L	W	Y	R	S	P	E
CDK2	P	V	R	T	Y	T	H	E	V	V	T	L	W	Y	R	A	P	E
GSK3	R	G	E	P	N	V	S	Y	I	C	S	R	Y	Y	R	A	P	E
ERK1	H	T	G	F	L	T	E	Y	V	A	T	R	W	Y	R	A	P	E
p38 alpha	T	D	D	E	M	T	G	Y	V	A	T	R	W	Y	R	A	P	E
JNK1	T	S	F	M	M	T	P	Y	V	V	T	R	Y	Y	R	A	P	E
PKC alpha	D	G	V	T	T	R	T	F	C	G	T	P	D	Y	I	A	P	E
PKC delta	G	E	S	R	A	S	T	F	C	G	T	P	D	Y	I	A	P	E
RSK1	H	E	K	K	A	Y	S	F	C	G	T	I	E	Y	M	A	P	E
MAPKAPK2	S	H	N	S	L	T	T	P	C	Y	T	P	Y	Y	V	A	P	E
CAMK1	P	G	S	V	L	S	T	A	C	G	T	P	G	Y	V	A	P	E
PIM1	K	D	T	V	Y	T	D	F	D	G	T	R	V	Y	S	P	P	E
AKT2	D	G	A	T	M	K	T	F	C	G	T	P	E	Y	L	A	P	E
PLK1	D	G	E	R	K	K	T	L	C	G	T	P	N	Y	I	A	P	E

Table 1. Alignment of phosphosites from activation loops of selected protein-serine/threonine kinases. Green, confirmed activation sites; yellow, confirmed phosphosites with unknown function; grey, potential phosphosites.

Results and Discussion

1. Kinase-dead mutant of ERK1

A kinase-dead (KD) mutant of ERK1 was created by mutating K71 of the ATP-binding subdomain II to alanine. Another mutant with the substitution of K72 to arginine was also constructed and expressed in *E. coli*. Purified GST-fusion ERK1 was assayed *in vitro* with wild-type (WT) MEK1 and a super active mutant MEK1-ΔEE (with deletion of its N-terminal auto-inhibition domain and double Glu mutation at two activatory phosphosites). ERK1's activity was tested by its ability to phosphorylate purified myelin basic protein (MBP) (Figure 2). The results showed a significant increase in phosphorylation by MEK1-ΔEE compared to MEK1-WT for all the three GST-ERK1 preparations. The K71A mutant of ERK1 lost most of its activity towards myelin basic protein (MBP), while the phosphorylation level of MBP by ERK1-WT and ERK1-K72R were comparable. Another interesting observation we had was the band shift after ERK1 phosphorylated by MEK1-ΔEE was lower in the KD K71A than with the other two with normal phosphotransferase activities, which may support the hypothesis that the flanking sites are autophosphorylated after the activation of ERK1 by MEK1 phosphorylation on the "TEY" site.

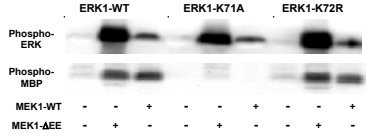

Figure 2. Phosphorylation of GST-ERK1 fusion proteins by MEK1 and their activities towards MBP.

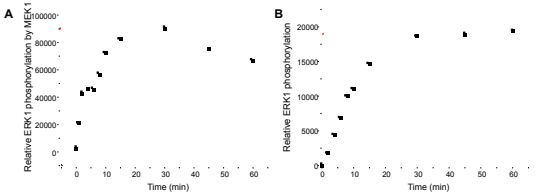

Figure 3. Time course experiment of ERK1 phosphorylation by MEK1-ΔEE. A. Relative phosphorylation level detected by phospho-ERK1 antibody against "TEY" sites. B. Relative phosphorylation of ERK1 in ^{33}P radioactive assay.

Table 2. Mutants of ERK1 on flanking phosphosites T198, T207 and Y210

ERK1	198										207			210				
WT	H	T	G	F	L	T	E	Y	V	A	T	R	W	Y	R	A	P	E
3A		A				T	E	Y			A			A				
2AF		A				T	E	Y			A			F				
AF		T				T	E	Y			A			F				
T198A		A				T	E	Y			T			Y				
T207A		T				T	E	Y			A			Y				
T207E		T				T	E	Y			E			Y				
Y210E		T				T	E	Y			T			E				

2. ERK1 Phosphorylation by MEK1-ΔEE

A time course experiment was performed using MEK1-ΔEE to phosphorylate ERK1-WT. The two purified kinases were incubated with ATP for up to 60 minutes. The phosphorylation was detected by ERK1 phospho-antibody specifically against the "TEY" sites as well as the ^{33}P radioactivity that represents the total phosphorylation level (Figure 3). By comparing the curves, we propose that the phosphorylation of the flanking sites are dependent on the "TEY" phosphorylation by MEK1, since the initial phosphorylation detected with both methods are similar. These additional sites may play a role in maintaining the overall phosphorylation level and phosphotransferase activity of ERK1.

3. Phosphorylation and activity of ERK1 mutants on the flanking phosphosites

The three flanking phosphosites of ERK1, T198, T207 and Y210 (position 147, 154 and 157 in the kinase domain alignment, respectively) were mutated individually or in combinations (Table 2), and expressed in bacteria as GST fusion proteins. So far we had the following observations from the two-step *in vitro* kinase assay of ERK1 with MEK1-ΔEE and MBP (Figure 4 and Figure 5):

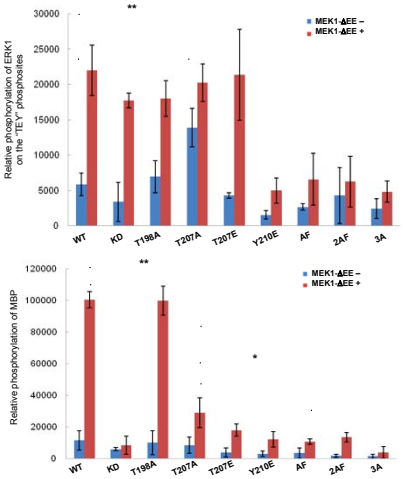

Figure 4. Phosphorylation of ERK1 mutants by MEK1-ΔEE (A) and their phosphotransferase activities towards MBP (B).

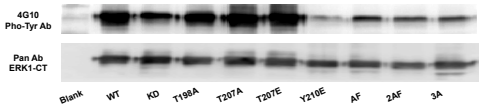

Figure 5. Tyrosine phosphorylation of ERK1 mutants by MEK1-ΔEE or autophosphorylation.

a. **Phosphorylation of the "TEY" phosphosites is insufficient for activation of ERK1.** Substitution of T207 to alanine significantly increased the autophosphorylation of the "TEY" phosphosites, but not the ability of ERK1 to phosphorylate MBP;
b. **Phosphorylation of T207, but not T198, plays an important role in the activation of ERK1.** Substitution of T207 to alanine significantly inhibited the ability of ERK1 to phosphorylate MBP without affecting the phosphorylation of "TEY" by MEK1;
c. **The tyrosine at position 210 is critical for the recognition of ERK1 by MEK1.** Mutation at this residue blocked the phosphorylation on "TEY" site as well as the phosphotransferase activity of ERK1. This could not be substituted with glutamic acid as a phosphosite mimetic;
d. **The phosphorylation of the flanking tyrosine depends on the ERK1 activity.** KD mutant of ERK1 has comparable level of phosphorylation on "TEY" site, but lower level of overall tyrosine phosphorylation (Figure 5) than WT. This supports the hypothesis that the flanking sites are autophosphorylated following the phosphorylation of "TEY" by MEK1.

Conclusion

From our results, we propose that the T207 and Y210 phosphosites of ERK1 become auto-phosphorylated after the phosphorylation of the "TEY" sites by MEK1, and this facilitates full activation of ERK1. Our findings contribute to an improved understanding of the activation of MAPKs and kinases in general. Hyper-phosphorylation within the kinase activation loop by autophosphorylation following the initial activation by other upstream kinases may be a common mechanism for protein kinases to achieve full stimulation.

Acknowledgement

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