International Journal of Mass Spectrometry 302 (2011) 101–109

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Distinct patterns of activation-dependent changes in conformational mobility between ERK1 and ERK2

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ARTICLE INFO

Article history:
Received 1 June 2010
Received in revised form 17 August 2010
Accepted 19 August 2010
Available online 15 September 2010

Keywords:
Hydrogen exchange
Mass spectrometry
ERK1
MAP kinase
Interdomain interactions

ABSTRACT

Hydrogen/deuterium exchange measurements by mass spectrometry (HX-MS) can be used to report localized conformational mobility within folded proteins, where exchange predominantly occurs through low energy fluctuations in structure, allowing transient solvent exposure. Changes in conformational mobility may impact protein function, even in cases where structural changes are unobservable. Previous studies of the MAP kinase, ERK2, revealed increases in HX upon activation occurred at the hinge between conserved N- and C-terminal domains, which could be ascribed to enhanced backbone flexibility. This implied that kinase activation modulates interdomain closure, and was supported by evidence for two modes of nucleotide binding that were consistent with closed *vs* open conformations in active *vs* inactive forms of ERK2, respectively. Thus, phosphorylation of ERK2 releases constraints to interdomain closure, by modulating hinge flexibility. In this study, we examined ERK1, which shares 90% sequence identity with ERK2. HX-MS measurements of ERK1 showed similarities with ERK2 in overall deuteration, consistent with their similar tertiary structures. However, the patterns of HX that were altered upon activation of ERK1 differed from those in ERK2. In particular, alterations in HX at the hinge region upon activation of ERK2 did not occur in ERK1, suggesting that the two enzymes differ with respect to their regulation of hinge mobility and interdomain closure. In agreement, HX-MS measurements of nucleotide binding suggested revealed domain closure in both inactive and active forms of ERK1. We conclude that although ERK1 and ERK2 are closely related with respect to primary sequence and tertiary structure, they utilize distinct mechanisms for controlling enzyme function through interdomain interactions.

1. Introduction

Extracellular signal-regulated protein kinases, ERK1 and ERK2, are mitogen-activated protein (MAP) kinases which control diverse cellular responses, including cell proliferation, differentiation, transformation, and survival. ERK activation is frequently dysregulated in human cancers, due to oncogenic mutations in upstream pathway components, including Ras, Raf, and MAP kinase kinase (MKK), as well as receptor tyrosine kinases, EGFR, Her2/Neu, and Met [1,2], all which are attractive targets for drug intervention. Although ERK1 and ERK2 are functionally redundant in many cell types, each shows a distinct mouse knockout phenotype, where deletion of ERK2 leads to embryonic lethality while deletion of ERK1 yields viable animals with defects in thymocyte maturation and immunodeficiency [3,4]. Thus, at least some functions of ERK1 and ERK2 are non-redundant. On the other hand, relatively little is known about how regulatory mechanisms controlling ERK1 and ERK2 differ, which might impact the development of different strategies for specifically targeting each form.

Crystallographic studies of ERK1 and ERK2 show a conserved tertiary structure shared with all protein kinases, consisting of an N-terminal ATP binding and C-terminal substrate binding domains surrounding the catalytic site [5–8]. Both ERK1 and ERK2 are activated by dual phosphorylation at a conserved pThr-Glu-pTyr sequence located within the activation lip region, which is catalyzed by the upstream MAP kinase kinases, MKK1 and MKK2. Structural remodeling events which accompany ERK activation have been revealed by X-ray structures of inactive, unphosphorylated (0P) and active, diphosphorylated (2P) forms of ERK2 [6–8]. Diphosphorylation triggers new phosphate-side chain ion pair interactions which lead to dramatic conformational changes within the activation lip, reorganizing the substrate binding site to enable recognition of the proline-directed phosphorylation motif (pSer/pThr-Pro), and reorienting active site residues involved in catalysis.

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doi:10.1016/j.ijms.2010.08.020

Hydrogen-exchange mass spectrometry (HX-MS) studies have shown that protein kinases are regulated not only through changes in conformation, but also through changes in conformational mobility. In previous studies of ERK2, comparisons between the active and inactive states of this kinase revealed increased HX in the hinge region upon activation, consistent with enhanced flexibility and conformer interconversions, which were not observable by X-ray crystallography [9]. Electron paramagnetic resonance (EPR) analysis of backbone residues in ERK2 that were individually coupled to a MTSL nitroxide spin label probe demonstrated that changes in side chain correlation rates occurred upon phosphorylation and activation [10]. Taken together, the results suggested a model in which the changes in HX could be explained by regulated protein motions, such as underlying backbone flexibility. Further measurements of steric protection from HX by the ATP analogue, AMP-PNP, suggested a function for this control of hinge flexibility. On one hand, HX within the N-terminal domain was comparable between 0P-ERK2 and 2P-ERK2, in regions containing the Gly loop, the conserved Lys-Glu ion pair within β3 and αC, the hinge region, and the catalytic base, all of which are known to form close interactions with nucleotide [11]. On the other hand, 2P-ERK2 showed 10-fold greater protection from HX than 0P-ERK2 within the conserved DFG motif, part of the C-terminal domain which forms metal coordination interactions with Mg^{+2}–ATP. Thus, AMP–PNP binding sterically protected both N- and C-terminal domains of 2P-ERK2 from solvent, but mainly protected only the N-terminal domain of 0P-ERK2. Because the two kinase forms shared similar binding affinities for AMP-PNP, the results suggested that distinct modes of nucleotide binding occur in solution. The protection patterns are consistent with a model in which 2P-ERK2 adopts a closed conformation, whereas 0P-ERK2 is somehow constrained to interfere with interdomain closure. Thus, HX protection by Mg^{+2}–AMP–PNP binding provides a useful way to monitor domain closure, an event needed to form a competent catalytic site. These results, together with the observed changes in hinge flexibility upon activation, led us to propose that activation of ERK2 releases constraints to domain closure by increasing backbone flexibility at the hinge [11].

Given their sequence and structural conservation, different MAP kinases might be expected to show similar regulatory mechanisms. In agreement, p38α MAP kinase appears to undergo conformational rearrangements following phosphorylation which parallel those seen in ERK2. For example, structural comparisons between p38α MAP kinase in its inactive, unphosphorylated form and the related p38γ MAP kinase in its active, diphosphorylated form show large conformational changes within the activation lip following phosphorylation by MKK3/6 [12–14]. Thus, although the activation lip conformation of 0P-p38α MAP kinase differs from that of 0P-ERK2, the lip conformation of 2P-p38γ MAP kinase is similar to that of 2P-ERK2, revealing that diverse activation lip conformers in MAP kinases converge towards a uniform structure in the active state, which allows catalytic rate enhancement. In contrast, HX-MS studies show that p38α MAP kinase differs significantly from ERK2 with respect to the patterns of regional HX which change in response to kinase activation [15]. This suggests that the effect of kinase activation on conformational mobility diverges between related protein kinases, leading to the hypothesis that different MAP kinases may have distinct mechanisms for regulating enzyme activity through their control of internal protein motions.

In this study, we wished to understand whether differences in patterns of regulated conformational mobility upon activation might also be observed between enzymes that are even more closely related. Here, we analyzed ERK1, which shares 90% sequence identity with ERK2. We found that ERK1 showed significant differences in how the HX behavior changes in response to kinase activation within conserved regions of the core structure. Notably, ERK1 did not display altered HX within hinge residues as previously observed in ERK2. This suggests that ERK1 might lack constraints to interdomain closure which are present in the inactive form of ERK2. In order to test this hypothesis, we measured changes in protection from HX upon AMP–PNP binding in ERK1, and compared these to prior studies of ERK2 [11]. The results showed significant protection from HX within the C-terminal domain of both 0P-ERK1 and 2P-ERK1, indicating that both inactive and active forms appear capable of interdomain closure in solution. Thus, the occurrence and functional effects of regulated protein motions differ in substantive ways between ERK1 and ERK2.

2. Materials and methods

2.1. Protein purification

Active, diphosphorylated (2P) ERK1 was produced from plasmid pET-His-ERK1-R4F, containing wild-type human His_6-ERK1 expressed in tandem with constitutively active mutant MKK1 (R4F: ΔN3/S218E/S222D) [16]. Inactive, unphosphorylated (0P) human His_6-ERK1 was produced using plasmid NpT7-ERK1 [17]. Each kinase form was expressed in *E. coli* strain BL21(DE3)-pLysS, and purified using Ni-NTA agarose (Qiagen) chromatography, desalted by gel filtration (PD10, GE Healthcare), and further purified by MonoQ FPLC. Proteins were dialyzed overnight into 50 mM KPO_4 (pH 7.4), 100 mM KCl and 5 mM dithiothreitol, and stored in aliquots at −80 °C. The 0P-ERK1 protein was found to be mono-phosphorylated to 0.13 mol/mol, and was therefore dephosphorylated using λ phosphatase (New England Biolabs, 100 U/μg, 3 h, 30 °C) before Mono Q separation. The activation lip phosphorylation stoichiometries of the final kinase preparations showed 0% phosphorylation in 0P-ERK1 at the regulatory Thr and Tyr residues in the activation lip, and 95% diphosphorylation in 2P-ERK1. Intact ESI-MS of 0P-ERK1 and 2P-ERK1 showed full length masses expected of the recombinant ERK1 sequences, in unphosphorylated and diphosphorylated forms (Supplementary Fig. S1).

2.2. HX-MS measurements

Data were collected on a QStar Pulsar QqTOF mass spectrometer (ABI) interfaced with an Agilent Cap1100 HPLC (0.5 mm i.d. × 10 cm column, packed with POROS R1 20 resin), and measurements of weighted average mass were performed as described [18,19]. Proteins (4 μg) were incubated in 90% D_2O (Sigma) at 10 °C, allowing in-exchange reactions to take place between 8 s and 4 h. Reactions were quenched with 90 μL 25 mM succinic acid, 25 mM citric acid (pH 2.4), and cooled rapidly to 0 °C. Proteins were digested by adding 10 μL pepsin (4 μg) and analyzed immediately by LC–MS. Time-zero measurements were performed by quenching the reaction before adding D_2O.

Peptides in pepsin digests of ERK1 were identified by LC–MS/MS, analyzing replicate digests on two instruments in order to maximize the probability that a peptide would be selected for MS/MS. Aliquots (4 μg) of proteolyzed ERK1 were analyzed on the QStar mass spectrometer, with m/z window 400–1600 Da, duty cycle 15.5 s, and three MS/MS per cycle. Aliquots (60 ng) of ERK1 were analyzed on an LTQ-Orbitrap mass spectrometer interfaced with an Eksigent 2DLC HPLC (75 μm i.d. × 150 mm column, Zorbax C18 resin), with m/z window 300–2000 Da, duty cycle 4–6 s (10–14 cycles/min), and five MS/MS per cycle. MS/MS were converted to .mgf files and searched against the human ERK1 sequence using MASCOT (v. 1.9), with no enzyme specified. Mass tolerances were 2.5 Da (parent) and 1.2 Da (fragment) for QStar datasets, and 1.2 Da (parent) and 0.6 Da (fragment) for LTQ-Orbitrap datasets. Peptides with MOWSE score greater than 20 were validated by manual analysis of MS/MS spectra. LC–MS of ERK1 was then carried