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Biochemical and molecular analysis of the interaction between ERK2 MAP kinase and hypoxia inducible factor-1α

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ABSTRACT

The mitogen activated protein kinase (MAPK) signaling pathways play significant roles in fundamental cellular processes, such as cell growth and differentiation. It has been shown that the specificity and efficacy of phosphorylation by MAP kinases rely upon distinct MAPK-docking domains (D-domains) found in a wide range of MAPK substrates including the ETS-transcription factor Elk-1. Importantly, the MAPK signaling cascade converges with the hypoxia-induced signaling pathway. The key regulator of hypoxia signaling is the heterodimeric transcription factor hypoxia inducible factor-1 (HIF-1). The α-subunit of HIF-1 (HIF-1α) is a substrate for the ERK2 MAP kinase. Unraveling the interplay of these main signaling systems is a prerequisite for understanding their role in tumor growth, a situation sustained by simultaneous mitogenic and hypoxic signals. In this work, we investigated the molecular cues that direct HIF-1α recognition and phosphorylation by ERK2. We showed that HIF-1α possesses a MAPK docking domain. Utilizing surface plasmon resonance (SPR) methodologies we demonstrated efficient binding between HIF-1α and ERK2, with a K_D value in the low micromolar range. Although, the D-domain did not contribute to the above interaction significantly, it could act *in trans* by recruiting ERK2 and conferring responsiveness to poor ERK substrates. These results indicate that, via its conserved D-domain, HIF-1α could serve as a platform for ERK2 in the nucleus of the cell, thus potentially facilitating phosphorylation of other ERK2 substrates. The identification of an ERK2 recognition domain on HIF-1α opens new avenues for the analysis of HIF-1α-related ERK2 signaling and may allow designing of interfering compounds.

1. Introduction

The mitogen activated protein kinase (MAPK) pathways are probably the best studied and characterized signaling systems [1–3]. They display high evolutionary conservation among eukaryotes, from yeast to *Drosophila* and humans. The signaling cascades rely on sequential phosphorylation of proteins and are organized in a three-kinase module. The terminal kinase (MAPK) is phosphorylated and activated by an upstream kinase or MAPK-Kinase (MAPKK), which in turn is phosphorylated and activated by a MAPKK-Kinase (MAPKKK). Activation of MAPKKK is a complex process that is also based on phosphorylation. The three major members of the MAPK family are the extracellular signal-regulated kinases (ERKs) 1 and 2, the c-Jun N-terminal kinases (JNKs) 1, 2 and 3, and the p38 isoforms α, β, γ and δ. Upon activation the MAPKs can either phosphorylate various cytoplasmic substrates such as cytoskeletal components, or translocate to the nucleus of the stimulated cell where they phosphorylate various transcription factors, thus promoting nuclear responses.

The MAPK pathways play a key role in regulating several fundamental processes, including cell growth, development, differentiation and apoptosis. Thus, precise regulation is critical for cell survival. Indeed, several forms of cancer and other diseases have been linked to aberrations within the MAPK signaling pathways

Abbreviations: MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; HIF-1α, hypoxia inducible factor-1α; DSF, differential scanning fluorimetry; SPR, surface plasmon resonance.

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[4]. In recent years, several mechanisms that maintain and control signal specificity and efficiency have been identified. Among them, distinct kinase docking sites on substrates play important role for the enhancement of the accuracy and efficacy of phosphorylation [5,6]. A typical docking domain termed D-domain, or DEJL motif, has been identified in various MAP kinase substrates, including the transcription factors c-Jun [7,8], Elk-1 [9], MEF2A, MEF2C [10] and SAP-1 [11], the MAPK/ERK kinases MEK1 and MEK2 [12] and the scaffold proteins JIP-1 and JIP-3 [13]. In general, the D-domain is a relatively short motif, approximately 14–20 residues long that lies far from the phosphoacceptor residues of the substrates both in the primary and the tertiary structure. The primary structure of a typical D-domain found in transcription factors is characterized by a basic region, a central LXL (L for leucine and X for any amino acid) motif and an N-terminal hydrophobic region. These three submotifs possess distinct properties and contribute differently in MAPK targeting as revealed by a detailed mutational analysis of the D-domains of the transcription factors SAP-1 and MEF2A [10,11]. It has been shown that the basic region is more important for the efficacy and fidelity of the interaction between the kinase and its substrate, whereas the residues of the hydrophobic motif play a critical role in specificity determination. Moreover, it has been demonstrated that targeting involves two distinct domains on the surface of the kinase. The common docking or CD domain is an acidic region located at the C-terminus, on a site opposite to the active center of the enzyme and contributes to kinase–substrate binding by interacting with the basic region of the D-domain [14], whereas the ED site, near the CD domain in the steric structure, confers docking specificity [15].

The MAPK signaling pathways are also involved in hypoxia response. It has been shown that ERK1/2 can directly phosphorylate the hypoxia inducible factor-1α (HIF-1α), the key regulator of hypoxia signaling, both *in vitro* and *in vivo* [16,17]. Mass spectroscopy revealed that serine residues at positions 641 and 643 are the two major phosphorylation sites [17]. It has been demonstrated that the phosphorylation does not affect HIF-1α protein stability nor DNA binding capacity, but it increases transcriptional activity of HIF-1α by controlling its subcellular localization [18]. Indeed, mutation of Ser641 and Ser643 renders HIF-1α mainly cytoplasmic, even during hypoxia, whereas the phosphomimetic mutation Ser641Glu promotes activity and nuclear accumulation. However, the molecular factors that control HIF-1α recognition and subsequent phosphorylation by ERK2 remain unknown. Elucidation of these parameters is critical to enhance our understanding of the regulatory mechanisms of hypoxia signaling with a profound impact on our efforts to develop novel therapeutics.

Here, we demonstrate that HIF-1α possesses a distinct MAPK docking domain. Using surface plasmon resonance (SPR) methodologies we determine equilibrium dissociation constants of ERK2 with HIF-1α and other substrates and evaluate the role of D-domain in ERK2 targeting. Our data show that HIF-1α D-domain acts *in trans* rather than *in cis*, by recruiting ERK2 and promoting substrate phosphorylation in a heterologous context.

2. Materials and methods

2.1. Plasmid construction

The following plasmids were constructed for the expression of GST-fusion proteins in *Escherichia coli*. GST-HIF-376 (HIF-1α amino acids 376–826) and GST-HIF-420 (HIF-1α amino acids 420–826) were generated by inserting BamHI cleaved PCR-derived fragments into the same site of pGEX-4T-1 bacterial expression vector (Amersham Biosciences). GST-HIF-376-m1, GST-HIF-376-m2, GST-HIF-376-m3 and GST-HIF-376-m4 were derived from GST-HIF-376 by site-directed mutagenesis that created the following point mutations respectively: K391A/K392A, L397A/L399A, I408A/I409A and L399A/L400A. GST-Elk-310 and GST-Elk-330 were kindly provided by Professor A. Sharrocks, University of Manchester, U.K. GST-HIFD-Elk-330 (HIF-1α amino acids 376–409 fused to Elk-1 amino acids 330–428) was generated by a two-step PCR protocol. The first step utilized GST-HIF-376 as template whereas the second step utilized GST-Elk-310. The resulting PCR fragment was BamHI/EcoRI cleaved and inserted into the same sites of pGEX-4T-1. Details of the mutagenic primers can be supplied on request. The full length HIF-1α cDNA (Origene) was BamHI cleaved and inserted into the same sites of pGEX-4T-1. For the expression of recombinant ERK2, the appropriate plasmid was constructed by ligating the BamHI-HindIII fragment from ERK2 cDNA (wild type) in pUSEamp (upstate biotechnology) into the same sites of pETDuet-1 expression vector (Novagen). All plasmid constructs made by polymerase chain reaction were verified by automated dideoxy sequencing.

2.2. Protein expression and purification

GST-fusion proteins were expressed in *E. coli* BL21 cells and purified as described previously [11]. Recombinant ERK2 was expressed in *E. coli* BL21(DE3) cells and purified following standard procedures. Briefly, cells were harvested and the pellet was resuspended in ice cold lysis buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 5 μg/ml pepstatin) followed by brief sonication. Insoluble debris was pelleted by centrifugation at 20,000 × *g* for 30 min at 4 °C. The resulting supernatant was mixed with Ni-NTA agarose beads (Qiagen) at 4 °C for 60 min under gentle rotation. The beads were washed firstly with 10 volumes of wash buffer A (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 20 mM imidazole), then with 10 volumes of wash buffer B (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 40 mM imidazole) and finally the bound proteins were eluted with elution buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 250 mM imidazole). The eluted protein was mixed with glycerol (final concentration 20% v/v) and stored at −20 °C until use. The purity and the quantity of the isolated recombinant proteins were examined by SDS polyacrylamide gel electrophoresis followed by Coomassie blue staining. The concentration of the recombinant proteins was determined meticulously by both Bradford assay and comparison with bovine serum albumin (BSA) as a standard after SDS-PAGE and Coomassie blue staining.

2.3. Protein kinase assays

The kinase assays were initiated by the addition of substrate protein (as specified), 5 mM ATP, 50 mM [γ-^{32}P]ATP (10 Ci/mmol) and recombinant active p42 MAPK (New England Biolabs, Beverly, MA) in kinase buffer (25 mM HEPES [pH 7.4], 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM sodium orthovanadate) in 20 μl (final volume). The reactions were carried out at 30 °C and terminated at the times specified by the addition of 5 μl of 5× Laemmli sample buffer. The phosphorylation of substrate proteins was examined after SDS-PAGE by autoradiography and quantified by phosphorimaging (Fuji BAS1500 phosphorimager; Tina 2.08e software).

2.4. Surface plasmon resonance spectroscopy

Interaction of ERK2 with the substrates was investigated using surface plasmon resonance spectroscopy. All binding experiments were carried out on Proteon XPR36 (Bio-Rad) biosensor at 25 °C