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Osmotic and heat stress-dependent regulation of MLK4β and MLK3 by the CHIP E3 ligase in ovarian cancer cells

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ABSTRACT

Mixed Lineage Kinase 3 (MLK3), a member of the MLK subfamily of protein kinases, is a mitogen-activated protein (MAP) kinase kinase kinase (MAP3K) that activates MAPK signalling pathways and regulates cellular responses such as proliferation, invasion and apoptosis. MLK4β, another member of the MLK subfamily, is less extensively studied, and the regulation of MLK4β by stress stimuli is not known. In this study, the regulation of MLK4β and MLK3 by osmotic stress, thermostress and heat shock protein 90 (Hsp90) inhibition was investigated in ovarian cancer cells. MLK3 and MLK4β protein levels declined under conditions of prolonged osmotic stress, heat stress or exposure to the Hsp90 inhibitor geldanamycin (GA); and MLK3 protein declined faster than MLK4β. Similar to MLK3, the reduction in MLK4β protein in cells exposed to heat or osmotic stresses occurred *via* a mechanism that involves the E3 ligase, carboxy-terminus of Hsc70-interacting protein (CHIP). Both heat shock protein 70 (Hsp70) and CHIP overexpression led to polyubiquitination and a decrease in endogenous MLK4β protein, and MLK4β was ubiquitinated by CHIP *in vitro*. In untreated cells and cells exposed to osmotic and heat stresses for short time periods, small interfering RNA (siRNA) knockdown of MLK4β elevated the levels of activated MLK3, c-Jun N-terminal kinase (JNK) and p38 MAPKs. Furthermore, MLK3 binds to MLK4β, and this association is regulated by osmotic stress. These results suggest that in the early response to stressful stimuli, MLK4β-MLK3 binding is important for regulating MLK3 activity and MAPK signalling, and after prolonged periods of stress exposure, MLK4β and MLK3 proteins decline *via* CHIP-dependent degradation. These findings provide insight into how heat and osmotic stresses regulate MLK4β and MLK3, and reveal an important function for MLK4β in modulating MLK3 activity in stress responses.

1. Introduction

The mixed lineage kinases (MLKs) are a family of serine and threonine mitogen-activated protein (MAP) kinase kinase kinases (MAP3Ks) that regulate MAP kinase (MAPK) signalling in response to mitogenic and stressful stimuli [1–6]. MAPK signalling pathways transduce signals from the plasma membrane and mediate specific transcriptional responses to a wide range of stimuli [7]. Activated MAP3Ks promote activation of MAPK kinases (MAP2Ks), which in turn phosphorylate and activate MAPKs in a phosphorelay module [7]. Activated MAPKs translocate to the nucleus and induce transcription of genes implicated in various cellular responses including proliferation, migration, motility, and differentiation.

The MLK family consists of three sub-families: the MLKs, the dual leucine zipper-bearing kinases (DLKs) and the zipper sterile α-motif kinases (ZAKs) [8]. Members of the MLK subfamily include MLKs 1–3, 4α and 4β, which all possess an N-terminal SH3 domain, a Cdc42-Rac interactive binding (CRIB) domain, a leucine zipper region, a catalytic kinase domain and a proline rich C-terminal region [8]. MLK3, the most extensively studied member of the MLK subfamily, has critical functions in migration and invasion in breast, gastric, and ovarian cancer cells; and MLK3 regulates neuronal cell apoptosis [9–12]. *MLK3* has > 70% sequence identity to the *MLK4* catalytic domain; however, recent studies indicate non-redundant functions of these enzymes in normal and

Abbreviations: MLK3, Mixed Lineage Kinase 3; MLK4, Mixed Lineage Kinase 4; MAPK, mitogen-activated protein kinase; MAP3K, MAPK kinase kinase; MAP2K, MAPK kinase; JNK, c-Jun N-terminal kinase; siRNA, short interfering RNA; ERK, extracellular signal-regulated kinase; HEK293, human embryonic kidney 293; DLK, dual leucine zipper-bearing kinase; ZAKs, zipper sterile α-motif kinases; CRIB, Cdc42-Rac interactive binding; Hsp90, heat-shock protein 90; Hsp70, heat shock protein 70; CHIP, carboxy-terminus of Hsc70-interacting protein; CHX, cycloheximide; GA, geldanamycin; GFP, green fluorescent protein

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neoplastic cells [3,4,13,14].

MLK4 is expressed in the liver, lung, brain, kidney and pancreas; and has two isoforms, *MLK4α* and *MLK4β*, generated by alternative splicing [13]. MLK4β negatively regulates basal as well as stimulus-induced MAPK signalling, ovarian cancer cell invasion, and matrix metalloproteinase 9 activity [3,4]. In response to TNFα and osmotic stress, MLK4β down-regulates p38 signalling, which was proposed to be mediated through binding and inhibition of MLK3 kinase activity [4]. MLK4 interacts with toll-like receptor 4 and suppresses lipopolysaccharide-induced c-Jun N-terminal kinase (JNK) and extracellular stimulus-regulated kinase (ERK) signalling [3]. Mutated MLK4 kinase has been detected in microsatellite stable colorectal tumours, and these mutant alleles have been characterized as being either loss-of-function or tumorigenic [14,15]. Thus, MLK3 and MLK4 are important regulators of cellular transformation, and effective targeting of these enzymes in tumor cells requires a thorough understanding of MLK protein level and kinase activity modulation.

Recent findings have shed light on the stress-induced degradation of MLK3 protein. MLK3 is associated with heat-shock protein 90 (Hsp90) in breast cancer cells [16]. Hsp90 functions as a stabilizing partner that preserves the native conformation and ligand binding sites of the client protein [17]. Geldanamycin (GA), an Hsp90 inhibitor, binds the ATP pocket on Hsp90 and blocks the Hsp90 ATPase activity [18]. The inhibition of ATPase activity causes Hsp90 to lose association with client proteins [19]. These proteins are then ubiquitinated and targeted for proteasomal degradation by an Hsp70-dependent mechanism [20]. Hsp70 recruits an E3 ubiquitin ligase, carboxy-terminus of Hsc70-interacting protein (CHIP), that ubiquitinates and marks target proteins for degradation by the 26S proteasome [17]. We have previously shown that MLK3 undergoes CHIP-mediated polyubiquitination and proteasomal degradation in response to GA, heat shock and osmotic shock [21]. In light of recent findings that MLK4 influences cellular responses important for tumor development, we sought to gain insight into the modulation of MLK4β protein in ovarian cancer cells. This study is focused on the regulation of MLK3 and MLK4β proteins and MAPK signalling in response to heat, osmotic stress and Hsp90 inhibition in ovarian cancer cells.

2. Materials and methods

2.1. Cell culture

Human SKOV3 and TOV112D ovarian cancer and human embryonic kidney 293 (HEK293) cells were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA. SKOV3, TOV112D and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 25 μg/ml streptomycin and 25 I.U. penicillin (Mediatech, Inc.). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Plasmid and siRNA transfections

The following mammalian expression plasmids for expression of human CHIP, MLK3, MLK4β and Hsp70 were used in this study: pCMV-FLAG-CHIP, pCMV-FLAG-MLK3, pCMV-His-MLK3 and pCMV-GST-MLK4β, pEBG-GST. pCMV-EGFP-Hsp70 was obtained from Addgene, Cambridge, MA, USA [22]. The plasmids that were used for the expression of human MLK3 and CHIP in *E. coli* were pCMV-His-MLK3, pCMV-GST-MLK4β and pCMV-GST-CHIP. Transient transfections were performed using PolyJet™ (SignaGen Laboratories, Rockville, MD, USA), and siRNA knockdown was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or GeneMute™ (SignaGen Laboratories) as described by the manufacturer. MLK4 siRNA oligos (Ambion, Life Technologies, Grand Island, NY, USA) have the following sequence: 5′-GGGCAGTGATGACTGAGAT-3′ which corresponds to nucleotides 1208–1227. The MLK3 siRNA oligo sequence was described previously [6]. The human CHIP and non-target siRNA oligos were from Santa Cruz Biotechnology, Dallas, TX, USA.

2.3. Cell treatments

Cells were either left untreated, treated with vehicle, treated with a final concentration of 10 μM GA (InvivoGen, San Diego, CA, USA), 50 μM cycloheximide (CHX) (Thermo Fisher Scientific, Rockford, IL, USA), 250 mM sorbitol, or cultured at 42 °C for the indicated time periods, as previously described [21]. The cells were harvested immediately after treatments and immunoprecipitations were performed or whole cell extracts were prepared.

2.4. Protein purification from bacterial and mammalian cells

Mammalian FLAG-CHIP, GST-MLK4β and His-MLK3 proteins were expressed in BL21 *E. coli* cells and purified as performed previously [21].

2.5. In vitro binding assay and immunoprecipitation

For binding assays, 1–2 μg of His-tagged or GST fusion proteins (purified from BL21 *E. coli* or expressed and purified from HEK293 cells) were incubated together in lysis buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 2 mM EGTA, 0.1% βMe, 50 mM NaF, 1 mM Na_3VO_4, 1 μM leupeptin, 1 μM aprotinin, 1 mM PMSF and 1 mM benzamidine) and rotated with 20 μl Ni-NTA-agarose (for His-pulldown) or GSH-agarose beads (for GST-pulldown) at 4 °C for 2 h. After incubation, the beads were washed with high stringency wash buffer (0.1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 0.1% βMe, 0.5 M LiCl, 50 mM NaF, 1 mM Na_3VO_4, 1 μM leupeptin, 1 μM aprotinin, 1 mM PMSF, and 1 mM benzamidine) and then with wash buffer (same as high stringency wash without LiCl). His-tagged and GST fusion protein levels were assessed by immunoblotting with the appropriate antibodies.

For immunoprecipitations, HEK293 cells were transfected with appropriate mammalian expression plasmids. 24 h post transfection the cells were lysed in 1 ml IP lysis buffer (supplemented with 10 mM *N*-ethylmaleimide when detecting ubiquitinated proteins). The lysates were incubated with FLAG (Agilent, Santa Clara, CA, USA), green fluorescent protein (GFP) (Novus Biologicals, Littleton, CO, USA), MLK3 (D-11, Santa Cruz Biotechnology, Santa Cruz, CA), MLK4 or ubiquitin (Novus Biologicals) antibodies and 25 μl Protein-G sepharose beads (Pierce, Thermo Fisher Scientific Inc.) at 4 °C for 2 h with rotation. The beads were collected and washed with high-stringency wash buffer (as described above) and then with wash buffer (as described above). The samples were boiled in SDS sample buffer and analyzed by immunoblotting.

2.6. Immunoblotting

Immunoblotting was carried out with the following primary antibodies: MLK3 (C-20), GST (Z-5), JNK1 (C-17), Hsp90 (H-114), β-Actin (C-4), and CHIP (H-231) (Santa Cruz Biotechnology). The activation-state phospho-JNK (p-JNK; Thr183/Tyr185), phospho-MLK3 (p-MLK3; Thr277/Ser281), phospho-ERK (p-ERK; Thr202/Tyr204), and phospho-p38 (p-p38; Thr180/Tyr182) were obtained from Cell Signaling Technology, Beverly, MA, USA. Other antibodies that were used for immunoblotting were GFP and MLK4 (Novus Biologicals), FLAG (Agilent), Ubiquitin (BD Biosciences, San Jose, CA) and Hsp70 (W-27) (Thermo Scientific, Freemont, CA, USA).

2.7. In vitro ubiquitination assay

Mammalian His-CHIP and GST-MLK4β proteins were purified from