Cellular Signalling 25 (2013) 429–438

Contents lists available at SciVerse ScienceDirect

Cellular Signalling

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

Differential regulation of M3/6 (DUSP8) signaling complexes in response to arsenite-induced oxidative stress

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ARTICLE INFO

Article history:
Received 20 April 2012
Received in revised form 7 November 2012
Accepted 8 November 2012
Available online 15 November 2012

Keywords:
Phosphatases
Protein interactions
DUSP8
JNK
JIP
Arsenite

ABSTRACT

Mitogen-activated protein kinase (MAPK) cascades are involved in the regulation of cellular proliferation, differentiation, survival, apoptosis, as well as in inflammatory responses. Signal intensity and duration have been recognized as crucial parameters determining MAPK signaling output. Phosphatases play a particularly important role in this respect, by tightly controlling MAPK phosphorylation and activation. M3/6 (DUSP8) is a dual-specificity phosphatase implicated in the dephosphorylation and inactivation of JNK and, to a lesser extent, p38 MAPKs and is found in a complex with these kinases, along with other pathway components, held together by scaffold proteins. The JNK family consists of three genes, giving rise to at least ten different splice variants. Some functional differences between these gene products have been demonstrated, but the underlying molecular mechanisms and the roles of individual splice variants are still incompletely understood. We have investigated the interaction of M3/6 with JNK isoforms, as well as scaffold proteins of the JNK interacting protein (JIP) family, in order to elucidate the contribution of M3/6 to the regulation of distinct JNK signaling modules. M3/6 exhibited stronger binding towards JNK1β and JNK2α isoforms and this was reflected in higher enzymatic activity towards JNK2α2 when compared to JNK1α1 in vitro. After activation of the pathway by exposure of cells to arsenite, the interaction of M3/6 with JNK1α and JNK3 was enhanced, whereas that with JNK1β or JNK2α decreased. The modulation of binding affinities was found to be independent of JNK-mediated M3/6 phosphorylation. Furthermore, arsenite treatment resulted in an inducible recruitment of M3/6 to JNK-interacting protein 3 (JIP3) scaffold complexes, while its interaction with JIP1 or JIP2 was constitutive. The presented data suggest an isoform-specific role for the M3/6 phosphatase and the dynamic targeting of M3/6 towards distinct JNK-containing signaling complexes.

1. Introduction

A subgroup of dual-specificity phosphatases (DUSPs) dephosphorylates and thereby inactivates mitogen-activated protein kinases (MAPKs), with at least 10 DUSPs targeting MAPKs identified in mammals to date [1,2]. The DUSP M3/6 (DUSP8, hVH5) is involved in the negative regulation of two of the major MAPK pathways by selectively dephosphorylating c-Jun N-terminal kinases (JNK) and to a lesser extent p38 [3]. JNK and p38 pathways are activated in response to cellular and environmental stress signals, such as reactive oxygen species (ROS), UV and ionizing radiation, but also by cytokines and growth factors. While the p38 pathway is mostly recognized for its role in the regulation of immune and inflammatory responses, JNK isoforms have mainly been associated with diverse cellular processes such as apoptosis, differentiation and cell proliferation [4]. Four p38 isoforms, termed p38α, β, γ and δ, have been identified in mammals and specific but also redundant functions based on their tissue distribution and substrate preference have been described [5]. The JNK family of MAPKs consists of three JNK genes (JNK1, 2 and 3) [6]. Alternative exon usage in the kinase domain generates JNK1α/JNK2α and JNK1β/JNK2β variants, while additional alternative splicing near the 3′ end of the coding region of all three JNK genes gives rise to short 45 kDa and long 54 kDa JNK gene products [7]. This leads to the existence of at least ten different splice variants, which exhibit varying affinities for their transcription factor substrates and distinct expression/activation patterns in different cell types and tissues, suggesting that the isoforms may target different subsets of JNK substrates [7–11]. Signal intensity and duration, as well as compartmentalization mediated by scaffold proteins, have been recognized as crucial for determining signaling output of specific JNK gene products [12–14]. Scaffold proteins, such as the JNK-interacting proteins JIP1, 2 and 3 link JNKs to a particular upstream signal by interacting with a specific set of JNK pathway activators of the JNK pathway. The three JIP isoforms and their splice variants show distinct tissue distributions and binding preferences for signaling molecules involved in the JNK pathway, each bringing together a specific combination of pathway components [15,16].

Abbreviations: JNK, c-Jun N-terminal kinase; DUSP, dual-specificity phosphatase; JIP, JNK-interacting protein; MAPK, mitogen-activated protein kinase; MKP, MAP kinase phosphatase.

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http://dx.doi.org/10.1016/j.cellsig.2012.11.010

Apart from facilitating signal propagation, JIP scaffolds are involved in negative regulation of the JNK pathway by interacting with the phosphatases MKP-7 and M3/6 [17]. Tight control of kinase activity is essential for achieving specificity in MAPK mediated signalling responses. JNK isoforms are activated by dual phosphorylation on a T–P–Y motif in the activation loop of the kinase, while p38 requires phosphorylation of a T–G–Y motif [4]. Several dual specificity phosphatases exhibit substrate specificity for JNK and p38 kinases and are able to dephosphorylate both the threonine and tyrosine residues in the phosphorylated motif [18,19]. As mentioned above, the dual specificity phosphatase M3/6 has been implicated in the dephosphorylation and inactivation of JNK and p38 kinase [3].

Regulation of the phosphatase activity is still incompletely understood but it has been demonstrated that M3/6 (hVH5) mRNA levels are up-regulated in response to a variety of stimuli activating JNK, such as nerve growth factor, insulin or phorbol ester, suggesting a negative feedback regulation [20,21]. Furthermore, M3/6 protein levels are negatively regulated by degradation via the ubiquitin pathway and protein aggregation [22,23], while we have previously shown that phosphorylation of M3/6, mediated by its substrate JNK, leads to modulation of M3/6 activity towards JNK [24].

Several lines of evidence have suggested that individual splice variants of the three JNK genes regulate distinct cellular functions, raising the question of how this differential control is modulated [9,25–30]. Numerous studies have been conducted investigating the roles of individual DUSPs in the regulation of JNK (for review see [19]), but the question of isoform specificity has not been addressed in detail so far. In this study we investigated the differential interaction of M3/6 with JNK isoforms and splice variants, as well as with JIP scaffold proteins, in order to gain a better understanding of the regulatory mechanisms by which M3/6 controls JNK activity. Protein complexes formed between JNK isoforms, p38 kinases, JIP proteins and M3/6 were analyzed. Co-precipitation studies showed that M3/6 exhibits distinct specificities towards JNK and JIP isoforms and their interaction can be differentially modulated by arsenite-induced oxidative stress, suggesting a specific and dynamic targeting of M3/6 towards distinct signaling complexes.

2. Materials and methods

2.1. Antibodies

Anti-FLAG M2-Antibody and rabbit anti-FLAG-antibody were obtained from SIGMA. Anti-c-Myc 9E10, anti-JNK2, and anti-JIP1 antibodies were from Santa Cruz. Anti-phospho-JNK antibody was purchased from Promega. Anti-M3/6 antibody was a kind gift by Dr. A. Ashworth. A goat anti-mouse (Southern Biotech) and a goat anti-rabbit (Santa Cruz) were used as secondary, HRP-coupled antibodies.

2.2. Cell culture

Human embryonic kidney 293T cells were maintained in DMEM containing 10% fetal calf serum and antibiotic mix (100 U penicillin, 100 μg/ml streptomycin) in a humidified atmosphere at 37 °C and 5% CO_2. All reagents and sera were obtained from Gibco/Invitrogen. The cells were grown to confluence and passaged every 3–4 days. Transfections were carried out following a standard calcium phosphate protocol. When Biotin-M3/6 plasmids were transfected, a pcDNA3HA-BirA plasmid encoding the *E. coli* biotin ligase BirA was co-transfected to facilitate in vivo biotinylation of the tagged M3/6 protein [31].

2.3. Plasmids

pCMV-flag-JIP1, pcDNA3-flag-JIP2, pcDNA3-flag-JIP3 and pcDNA3-T7-JIP1ΔJBD as well as pcDNA3-flag-JNK and p38 plasmids were kind gifts of Dr. R. Davies (Howard Hughes Medical Institute, University of Massachusetts Medical School). The pcDNA3 flag-MKK7B2Jnk1α1 (APF) and pcDNA3 flag-MKK7B2Jnk1α1 plasmids were obtained from Addgene (plasmids 19730 and 19726, respectively). The HA-BirA cDNA and the biotinylation-signal cDNA were provided by Dr. J. Strouboulis (B.S.R.C. "Alexander Fleming") and have been described elsewhere [31]. The Bir A coding sequence, harboring an N-terminal hemagluttinin (HA) tag, was excised (EcoRI/XhoI) from pGEM-SD2 and ligated into pcDNA3.1C vector (Invitrogen). To create the pcDNA3.1hygroBiotin-M3/6 constructs, the M3/6 coding sequence (wild type or phosphatase inactive Cysteine to Serine mutant) was subcloned into a pcDNA3.1hygro vector (Invitrogen) using EcoRI and XhoI restriction sites. The cDNA sequence corresponding to the 23 amino acid biotinylation signal was amplified by PCR (PCR primers: 5′-AGTCGAGTTTACGGATCCCTATCAG-3′ and 5′-CCTGGAAG TCCATGGACTCATGC-3′) and subcloned in frame into the BamHI site of the pcDNA3.1hygro vector and an NcoI site spanning the start codon of the M3/6 coding sequence. pmT-SM M3/6 wild type and CS-mutant have been described [24]. The pmT-SM M3/6δ-deletion and the pmT-SM M3/6ΔJBR vectors were generated by two consecutive rounds of PCR. In brief, for the first PCR round, complementary oligonucleotides producing the deletion of interest were used in PCR reactions paired with either a forward M3/6 oligonucleotide or a reverse M3/6 oligonucleotide as appropriate, to generate 5′ and 3′ mutagenic products, respectively. A vector carrying the wild-type Myc-M3/6 sequence was used as template. The complementary mutagenic primers used were: M3/6δ-deletion: 5′-GTGTTGGCCTGACC GGCTCTCAGAAAG-3′ and 5′-CATCTTTCTGAGAGCCGGTCAGGCCAA CAC-3′ causing a deletion of amino acids 163 to 170 in the M3/6 coding sequence, M3/6ΔJBR: 5′-GCCTCCAGGACACCAACAAGACGGCCTA TGCACC-3′ and 5′-GGTGCATAGGCCGACTTGTTGGTGTCCTGGAGGC-3′ deleting amino acids 391 to 400 in the M3/6 coding sequence. The JNK2_1 chimera was constructed by introducing BglII restriction sites into the JNK1α2 and JNK2α2 sequences at the end of protein kinase subdomain IX, corresponding to amino acid 208 in JNK1α2. The Phusion® Site-Directed Mutagenesis Kit (NEB/Finzymes) was used according to the recommendations of the manufacturer and primers 5′-CAAGGAAAACGTAGATCTATGGTCTGTGG-3′ and 5′-TAGCCCATG CCAAGGATGACC-3′ for the pcDNA3FLAG-JNK1α2 template and 5′-CAAAGAGAACGTAGATCTCTGGTCAGTG-3′ and 5′-TAGCCCATACCC AGGATGACTTC-3′ for the pcDNA3FLAG-JNK2α2 template were used. Subsequently, both resulting vectors were digested with BglII and the 5′ fragment of the mutated JNK2α2 was subcloned into the BglII cut pcDNA3FLAG-JNK1α2 (BglII) vector, yielding a vector encoding a protein carrying the N-terminal 207 amino acids of JNK2α2 and the remaining C-terminal part starting from amino acid 208 of JNK1α2 (JNK2_1).

2.4. Cell lysis and protein pull-down experiments

Cells were lysed in 1% Tx-buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2 mM DTT and "Complete®" protease inhibitor (Roche)) and lysates were cleared by centrifugation. Expressed M3/6 protein containing a biotinylation signal tag was precipitated using Neutravidin® agarose (Thermo) by incubating the lysates with the affinity resin for 2 h at 4 °C under constant agitation. Following incubation, the agarose beads were washed three times using lysis buffer and Laemmli sample buffer was added to the sample for subsequent SDS-PAGE and Western blot analysis. For blotting, proteins were transferred to Protran nitrocellulose membrane (Whatman) and the ECL Plus reagent (GE Healthcare) was used for antibody detection on a STORM860 phosphor-imager system (Molecular Dynamics/GE Healthcare). Immunoprecipitation with either FLAG or Myc-antibodies was carried out by incubating lysates with the appropriate antibody for 5–16 h at 4 °C under constant agitation. Protein G sepharose (Zymed Laboratories) was subsequently