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Phosphorylation of the M3/6 dual-specificity phosphatase enhances the activation of JNK by arsenite

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ABSTRACT

Specific outcomes upon activation of the c-Jun N-terminal kinase (JNK) pathway critically depend on the intensity and duration of signal transmission. Dual-specificity phosphatases (DUSPs) play a very important role in these events by modulating the extent of JNK phosphorylation and activation and thus regulating cellular responses to stress. M3/6 (DUSP8) is one of the dual-specificity protein phosphatases with distinct specificity towards JNK. It has been shown that M3/6 itself is phosphorylated by JNK upon stimulation with arsenite, but the role of this phosphorylation has not been investigated. In this study, we mapped JNK-induced phosphorylation sites on M3/6 using mass spectrometry. Phosphorylated residues Ser 515, Thr 518 and Ser 520 were identified and site-directed mutagenesis was employed to investigate their role. Upon arsenite stimulation, M3/6 mutated at these sites exhibited decreased phosphorylation compared to the wild-type protein. No difference was observed in terms of the enzyme's *in vitro* phosphatase activity, its substrate specificity towards JNK isoforms, its interactions with JNK and the scaffold family of JNK-interacting proteins (JIPs), its stability or its subcellular localization. Interestingly, expression of M3/6 phosphorylation mutants delayed the time-course of JNK phosphorylation and activation by arsenite. We propose that phosphorylation of the M3/6 phosphatase by JNK in response to stress stimuli results in attenuation of phosphatase activity and acceleration of JNK activation.

1. Introduction

The mitogen-activated protein kinase (MAPK) signal transduction pathways play an essential role in the translation of environmental stimuli to appropriate biological responses, inducing proliferation, differentiation, inflammation, cell survival and apoptosis [1–5]. The transduction of the extracellular signal is carried out through a three-level cascade of kinases that sequentially phosphorylate each other, leading to the translocation of MAPKs to the nucleus and phosphorylation of specific transcription factors to affect gene expression [1,6].

It is becoming increasingly apparent that the magnitude and duration of MAPK activation dictate the specificity of signal transmission and determines the ultimate physiological response of the cell. A highly complex signaling-transcription feedback loop has recently been proposed as a general mechanism for discrimination between different signals [7]. Low level versus high level of MAPK activity, as well as transient versus sustained MAPK activation, can elicit entirely different cellular responses [8–10]. It is therefore essential that the extent of MAPK phosphorylation be tightly regulated and this can be achieved by the action of phosphatases, which are key enzymes in the signal transduction pathway. Dephosphorylation of either residue in the T–X–Y motif of MAPK results in inactivation of the kinase. It follows that three different types of phosphatase can inactivate MAPKs: serine/threonine phosphatases, protein tyrosine phosphatases, and phosphatases which can dephosphorylate the T–X–Y motif on both residues. The latter are called MAP kinase phosphatases (MKPs) and belong to the large family of dual-specificity phosphatases (DUSPs), thus called due to their ability to dephosphorylate both threonine and tyrosine residues. There is a high level of complexity involved in the mode of action of MKPs, as some show substrate specificity and preferentially inactivate a specific class of MAPK while others show broader specificity [11].

Stress-activated protein kinases (SAPKs), comprising of the p38 proteins (α,β,γ,δ) and the c-Jun N-terminal kinases (JNK1, JNK2, JNK3) are one of the major MAPK subfamilies. They are activated by a variety of stress stimuli, such as heat shock, radiation, oxidative and osmotic stress, as well as inflammatory cytokines [12,13]. This explains why JNK is associated with the pathogenesis of a variety of human diseases, including neurodegenerative, inflammatory and metabolic disorders, as well as cancer [14,15]. Arsenite, an environmental

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

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pollutant and a highly potent carcinogen, is one of the stress stimuli that activate the JNK pathway. It is not exactly clear how arsenite causes the activation of the JNK pathway; however, it is thought to have a dual effect on JNK activation, through the stimulation of JNK upstream kinases sensitive to the production of reactive oxygen species and also through the direct inactivation of JNK phosphatases [16–18]. There are several phosphatases that act on SAPKs, including MKP-1 (DUSP1), MKP-5 (DUSP10), MKP-7 (DUSP16) and M3/6 (DUSP8, human homologue also called hVH-5) [11,19]. The M3/6 dual-specificity phosphatase is a key regulator of the JNK pathway, as it has been shown to specifically dephosphorylate and inactivate the JNK MAPKs [20].

A very interesting characteristic of the M3/6 phosphatase is that upon arsenite activation of the JNK pathway, M3/6 itself is phosphorylated by JNK [21]. It has already been shown that various MKPs can be phosphorylated by their own MAPK substrates. For example, the MKP-1, MKP-2 and MKP-3 phosphatases which show activity towards the ERK MAPKs, as well as MKP-7, are phosphorylated by ERK following stimulation of the ERK pathway by growth factors or DNA damaging agents. Depending on whether a sustained activation or a decrease in the activation of the ERK pathway is involved, phosphorylation of these phosphatases can lead to their targeting for degradation or to their stabilization, respectively [22–28]. In contrast, a recent study on the function of DUSP5 phosphorylation by its substrate ERK has shown no effect on DUSP5 stability or activity [29].

M3/6 phosphorylation is specifically due to JNK activity, since p38- or ERK-specific inhibitors showed no effect on the phosphorylation of M3/6 after stimulation with arsenite [21]. In addition, we have shown that after stimulation with arsenite M3/6 is the only JNK phosphatase that is phosphorylated. However, the physiological role of this phosphorylation is not known. The aim of this study was to investigate the significance of M3/6 phosphorylation, its effect on the activity of the phosphatase and its importance for JNK signaling. We have mapped novel phosphorylation sites, generated M3/6 phosphorylation mutants and assayed their function in JNK activation upon arsenite stimulation. Our results show that phosphorylation of M3/6 results in the enhancement of JNK signaling.

2. Materials and methods

2.1. Phosphopeptide mapping

293T cells were transiently transfected with a wild-type pMT-SM myc-M3/6 construct (see below) and 48 h post-transfection were either stimulated with arsenite or left untreated. M3/6 was immunoprecipitated using a myc antibody and the immunoprecipitates were resolved on an SDS-PAGE gel. The gel was stained using a mass spectrometry-compatible silver nitrate protocol [30]. The phosphorylated M3/6 band from the arsenite-treated lysate was excised, destained and subjected to in-gel digestion with the endoproteinase AspN (Calbiochem). The in-gel digestion was performed with 12.5 ng AspN/μl in 40 mM ammonium bicarbonate buffer at 37 °C overnight. The resulting peptides were extracted and separated on a PepMap reversed phase C18 column (75 μm × 15 cm, LC Packings) using a nano-high performance liquid chromatography system (Ultimate, LC Packings, Sunnyvale, CA). The injected samples were eluted at 180 nl/min with a 2–80% (v/v) acetonitrile/water gradient containing 0.1% formic acid over 55 min. The MS analysis was performed on an LCQ Deca ion trap mass spectrometer (Thermo Electron Corporation, Inc. Waltham) fitted with a nanoESI source. For the analysis of peptides, data-dependent MS/MS experiments were performed. The method consisted of an MS scan, a zoom scan, and MS/MS of the most abundant ion from the parental MS scan. The data were collected using the Xcalibur software (Thermo Electron Corporation, Inc. Waltham) and peptide analysis was performed with the TurboSEQUEST software. The IPI mouse specific database was used for the search. Differentially modified serine and threonine molecular mass values were used to identify phosphorylated peptides (addition of 79.9 Da on serine and/or threonine). Data dependent MS^3 was performed to increase sequence coverage. Normalized collision energy was 50% for both MS^2 and MS^3.

2.2. Site-directed mutagenesis

The Ser515 → Ala, Ser520 → Ala and the Thr518 → Ala point mutations on M3/6 were generated by two consecutive rounds of PCR. In brief, for the first PCR round, complementary oligonucleotides carrying the mutation/s 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 pBluescriptKS vector carrying the wild-type myc-M3/6 sequence was used as template. The complementary mutagenic primers used were:

Ser515 → Ala, CACTGGACGCCCCAGGCAC (forward) and GTGCCTGG GGCGTCCAGTG (reverse);
Ser520 → Ala, CAGGCACACCGGCGCCCGAC (forward) and GTCGGG CGCCGGTGTGCCTG (reverse);
Ser 515/520 → Ala, CACTGGACGCCCCAGGCACACCGGCGCCCGAC (forward) and
GTCGGGCGCCGGTGTGCCTGGGGCGTCCAGTG (reverse);
Ser515 → Ala/Thr518 → Ala/Ser520 → Ala, CCGCCACTGGACGCCC CAGGCGCACCGGCGCCCGACGGCC (forward) and GGCCGTCGGG CGCCGGTGCGCCTGGGGCGTCCAGTGGCGG (reverse).

The external M3/6 forward primer – used in conjunction with the reverse mutagenic primers – corresponds to nucleotides 1185–1204, and the external M3/6 reverse primer – used in conjunction with the forward mutagenic primers – corresponds to nucleotides 1730–1748. For the second round of PCRs, the mutagenic 5′ and 3′ PCR products from the first round were used as templates in PCR reactions using the external M3/6 forward and reverse primers. The PCR products from these reactions were excised and purified using the QIAquick gel extraction kit (Qiagen), digested with BssHII enzyme, purified and subcloned into the BssHII site of the myc-M3/6 pMT-SM vector. All constructs were verified by DNA sequencing. The Pfu DNA polymerase was from Fermentas and the T4 DNA ligase was from Roche. Restriction endonucleases were supplied by New England Biolabs and Fermentas. All primers were supplied by MWG Biotech.

2.3. Cell culture and transfection

Human embryonic kidney 293T cells were used for all experimental procedures. Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For serum starvation, cells were incubated overnight in 0.1% fetal bovine serum. Cells were cultured at 37 °C in a humidified 5% CO2 atmosphere. All media and components were obtained from Gibco. Transient transfections were carried out using a standard calcium phosphate protocol. The plasmids used for transfections were as follows: The pMT-SM vector was provided by Dr. A. Ashworth (Institute of Cancer Research, London). All the M3/6 constructs were subcloned into the pMT-SM vector [31]. The pMT-myc-MKP-3 and pSRa-myc-MKP-7 plasmids were kind gifts of Dr. S. Arkinstall (Serono Reproductive Biology Institute, Massachusetts). The pMT-myc-MKP5 plasmid has previously been described [32]. The pSRa-HA-JNK2 plasmid as well as the pCMV-flag-JIP1 and pCDNA3-flag-JIP2 plasmids were provided by Dr. R. Davies (Howard Hughes Medical Institute, University of Massachusetts Medical School). The Rc-CMV-flag-JNK1 and the pMT-HA-JNK3/SAPKβ plasmids were obtained from Dr. C.J. Marshall (Institute of Cancer Research, London) and the RSV-c-Jun plasmid from Dr. A. Pintzas (National Hellenic Research Foundation, Athens). The pCDNA3 flag-MKK7B2Jnk1a1 (APF)