Biochemical and Biophysical Research Communications 432 (2013) 683–688

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

JNK1β1 is phosphorylated during expression in *E. coli* and *in vitro* by MKK4 at three identical novel sites

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ARTICLE INFO

Article history:
Received 23 January 2013
Available online 12 February 2013

Keywords:
MAP kinases
c-Jun N-terminal kinase
MKK4
Recombinant protein expression
Protein phosphorylation
Mass spectrometry

ABSTRACT

JNK1 is activated by phosphorylation of the canonical T183 and Y185 residues, modifications that are catalysed typically by the upstream eukaryotic kinases MKK4 and MKK7. Nonetheless, the exact sites at which the most abundant JNK variant, JNK1β1, is further modified by MKK4 for phospho-regulation has not been previously investigated. Aiming to characterise the nature of JNK1β1 phosphorylation by active MKK4 using mass spectrometry, a recognised yet uncharacterised phospho-site (S377) as well as two novel phospho-residues (T228 and S284) were identified. Interestingly, the identical sites were phosphorylated during overexpression of JNK1β1 in *Escherichia coli*, raising important questions that have significant implications for heterologous protein expression.

1. Introduction

The c-Jun N-terminal kinases (JNKs) constitute one sub-group of the eukaryotic mitogen activated protein kinases (MAPKs) that mediate the cellular response to an array of extracellular stimuli, including cytokines and stresses such as UV irradiation [1]. Upon activation by these stimuli, the multifunctional JNK pathway initiates a variety of transcription events implicated in many physiological processes that include differentiation, apoptosis, and DNA repair [1].

Three JNK isoforms have been identified; JNK1, JNK2 (both with broad tissue distribution), and JNK3 (localised predominantly in the CNS) [1,2]. These kinases are encoded by three alternatively spliced genes, (*JNK1*, *JNK2*, and *JNK3)*, resulting in 10 distinct JNK splice variants that differ in the length of their N- and C-termini, as well as in amino acids within and around their substrate binding sites [2].

JNKs are typically activated by a cascade of MAPK-mediated phosphorylation events. Within this highly conserved three-tiered kinase cascade, an upstream MAP3K (e.g. MEKK1) phosphorylates a MAP2K (e.g. MKK4 and MKK7), which in turn phosphorylates and activates the downstream MAPKs (e.g. JNK, ERK, and p38) [3]. Phosphorylation of the canonical T183/Y185 residues in the activation loop of JNK1 primes the enzyme for activities that include the phosphorylation of the transcription factors c-Jun and ATF2 [1,2], as well as interaction with several non-substrate, regulatory proteins [4]. T183/Y185 phosphorylation is typically catalysed by the dual-specificity MAP2Ks, MKK4 and MKK7, respectively [5] yet it has been shown that high concentrations of either kinase, once activated by upstream MAP3Ks, is sufficient to phosphorylate both residues [1,5].

In addition to the phosphorylation of its activation residues, which are topologically conserved across the JNK family (Supplementary Fig. S1), the kinase has been found to be phosphorylated at a variety of additional S/T/Y residues distributed throughout its structure (Supplementary Table S1 reports all JNK phospho-residues identified up to date). Unfortunately, the majority of these possible phosphorylation sites have been assigned using only proteomic-based discovery-mode mass spectrometry (MS), leaving their functional relevance and the upstream kinases that catalyse their phosphorylation uncharacterised. Few additional JNK phospho-sites have been determined unequivocally using site-specific methods such as specific MS strategies, amino acid sequencing, phosphorylated site-specific antibodies, and site-directed mutagenesis (Table S1). Many of these additional sites are also not conserved across the JNK family, suggesting that they may represent a means of differential phospho-regulation of the JNK variants, and may be responsible for the diversity in the function of the individual JNKs. Although the mechanism by which the JNKs are regulated

Abbreviations: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEKK, mitogen-activated ERK-regulating kinase kinase; MKK, mitogen-activated protein kinase kinase; ATF2, activating transcription factor 2; LC–MS, liquid chromatography mass spectrometry; wt-, wild-type; pp-, dual phosphorylated.

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.02.018

in the cell by phosphorylation before and immediately after stress in still not fully understood, the involvement of JNKs in various neurodegenerative and other pathological disorders makes these kinases attractive therapeutic targets [6], and stress the need to fully characterise the significance of the non-canonical phosphosites.

In this study, we explored the phosphorylation events of the most abundant yet least studied JNK variant, JNK1β1 [7], that take place during its heterologous expression in *Escherichia coli* and that are catalysed by active MKK4 *in vitro*. We thus aimed to characterise the nature of phospho-regulation of JNK1β1 by MKK4, since the exact effect that MKK4 has on this JNK variant has not been specifically investigated. Intact protein LC–MS analysis and peptide sequencing was performed to track the phosphorylation sites and patterns of the various forms of JNK1β1 obtained, resulting in the unambiguous identification of novel sites at which MKK4 phosphorylates JNK1β1. ATF2 phosphorylation was used as an index of the catalytic activity of the JNK1β1 phospho-forms. The surprising patterns of JNK1β1 phosphorylation observed here upon its purification from *E. coli* raise important questions that have significant implications for heterologous protein expression.

2. Materials and methods

2.1. Purification and modification of the JNK MAPK phosphorylation cascade proteins

Overexpression and purification of JNK1β1 from *E. coli*, dephosphorylation and deactivation of *E. coli* phosphorylated JNK1β1 by shrimp alkaline phosphatase (SAP), activation of JNK1β1 by reconstituting the MEKK1 → MKK4 → JNK MAPK phosphorylation cascade *in vitro*, as well as the purification of ATF2 (residues 17–96), were performed as previously described [8].

2.2. Additional methods

Methods for the mass spectrometric analysis and sequencing of the JNK1β1 phospho-forms, as well as for determining their relative catalytic activities, are described in Supplementary Methods.

3. Results and discussion

Recently, we have described an improved method to obtain large quantities of active, phosphorylated JNK1β1 [8]. This method aimed first to improve the soluble overexpression of JNK1β1 by harmonising the codon usage frequencies of its open reading frame to those of an *E. coli* expression host (full "codon usage harmonisation" [9]). We were thus able to recreate the natural rate at which JNK1β1 is translated in its native human host as a precautionary measure toward increasing the production of soluble, correctly folded protein in *E. coli*. Purified JNK1β1 was activated by reconstituting the MEKK1 → MKK4 → JNK MAPK phosphorylation cascade *in vitro*; MEKK-C (the constitutively active kinase domain of MEKK1) was used to activate MKK4, which in turn was used to phosphorylate and activate JNK1β1. MKK4 activated JNK1β1 phosphorylated its substrate, ATF2, with high catalytic efficiency [8].

To expand upon the successes of our previous study, we sought to characterise the nature of JNK1β1 phosphorylation by active MKK4, particularly since the efficiency and the precise sites at which the β1 variant of JNK1 is modified for regulation by MKK4 has not been investigated.

MKK4 binds to each JNK isoform selectively, interactions that are mediated by the docking-domains and common docking-domain of MKK4 and JNK, respectively [10]. The affinity of these interactions exhibit isoform-specificity, with JNK1 displaying the greatest specificity for its cognate docking-domain in MKK4 [10]. It has therefore been suggested that MKK4 may have distinct affinities and activities toward the different JNK1 splice variants. Since it has been realised that the effects of MKK4 are splice-variant specific, it becomes significant to characterise the nature of its phosphorylative regulation of each individual JNK variant. Additionally, although they have been shown to display distinct biochemical properties, little is known about the functional relevance of the different JNK slice variants. Studying the individual JNK variants thus expands our understanding of their differences, and will hopefully lead to elucidating their functional diversity. This was achieved in this study using mass spectrometric techniques and enzymatic analysis to identify the states and sites of phosphorylation of the resulting JNK1β1 phospho-forms described in [8].

Intriguingly, our pursuit to acquire and phosphorylate JNK1β1 revealed that the kinase undergoes a significant degree of phosphorylation during overexpression in *E. coli* (even before JNK1β1 had encountered the components thought necessary for its activation) (Supplementary Fig. S2) [8]. We thus used similar tools to provide insight into the means by which JNK1β1 is already partially phosphorylated in *E. coli* without additional *in vitro* treatment, stressing important implications for recombinant expression of kinases in *E. coli*. Therefore, using intact LC–MS protein analysis, we determined the relative populations of the JNK1β1 phospho-forms to provide an indication of the efficiency at which it is phosphorylated. Peptide sequencing was then performed to identify the amino acid locations that are phosphorylated during JNK1β1 overexpression and *in vitro* by MKK4.

3.1. JNK1β1 phosphorylation during expression in E. coli

Intact LC–MS analysis of the untreated JNK1β1 purified from *E. coli* (p*JNK1β1) confirmed that the kinase had indeed been phosphorylated during overexpression in the bacterium (profile 1, Fig. 1). Although the majority of p*JNK1β1 had not been phosphorylated (74%), its mass profile revealed unexpectedly that a small percentage of JNK1β1 (8%) had been phosphorylated in *E. coli* at no less than 4 sites, with concurrently slightly larger populations of singularly (11%) and doubly (7%) phosphorylated kinase existing after expression (Fig. 1).

Peptide sequencing following trypsin digestion of p*JNK1β1 revealed that it was in fact phosphorylated at five discrete sites (Fig. 2; see Supplementary Figs. S4 and S5 for the theoretical trypsin cleavage pattern of JNK1β1 and the high-confidence MS/MS product ion spectra of the resulting phospho-peptides, respectively). Despite being detected in relatively low abundance, corresponding to the small population of each p*JNK1β1 phospho-species (profile 1, Fig. 1), four unambiguous phospho-peptides were detected with >99% confidence (Fig. 2). The canonical JNK T183/Y185 residues were located on the same peptide due to their proximity and properties preventing the generation of peptides that contained each residue separately. Nevertheless, the T183/Y185 phospho-peptides detected were unambiguously and consistently phosphorylated at both residues (Supplementary Fig. S5). The other phospho-peptides identified each contained a single phosphorylated residue, revealing that JNK1β1 is also phosphorylated by some means at T228, S284, and S377 during expression in *E coli* (Fig. 2). While these phospho-sites may initially be considered nothing more than artefacts common to the recombinant overexpression of kinases, the significance of these residues became strikingly evident when determining the sites at which active MKK4 phosphorylates JNK1β1 (discussed below).

To determine if the phosphate groups added to the activation (T183/Y185) and additional residues (T228/S284/S377) during expression induce activation of JNK1β1, and to approximate the

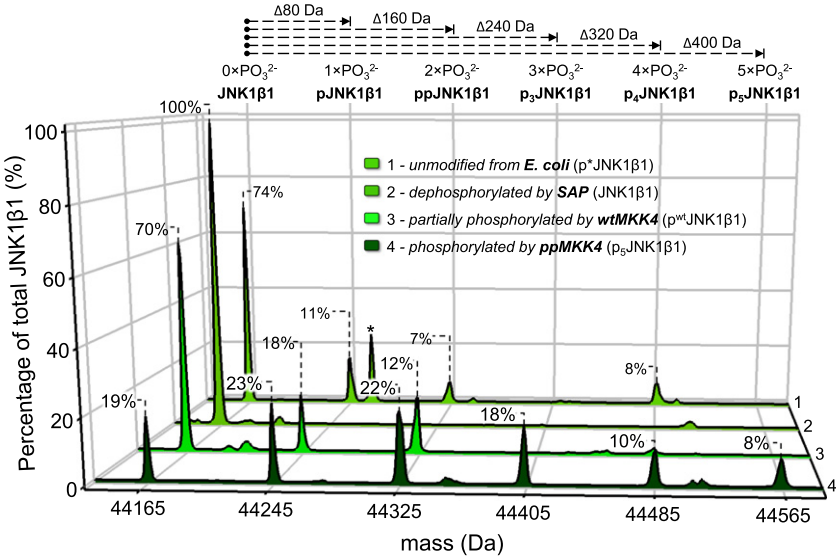

Fig. 1. The extent of phosphorylation of each JNK1β1 phospho-form, determined by intact protein LC–MS analysis. Intact protein mass profiles were generated by transforming the deconvoluted mass spectra of each phospho-form (Supplementary Fig. S3) so that the intensities of each JNK1β1 mass peak are expressed as a percentage of the total JNK1β1 present. This was calculated using the area under a particular peak relative to the total area under all JNK1β1 mass peaks. Non-phosphorylated JNK1β1 has a modal mass of 44 165 Da. The additional mass species increasing by multiples of 80 Da correspond to the addition of up to 5 phosphate groups. Profiles are representative of at least two independent experiments. "*" denotes likely contaminants.

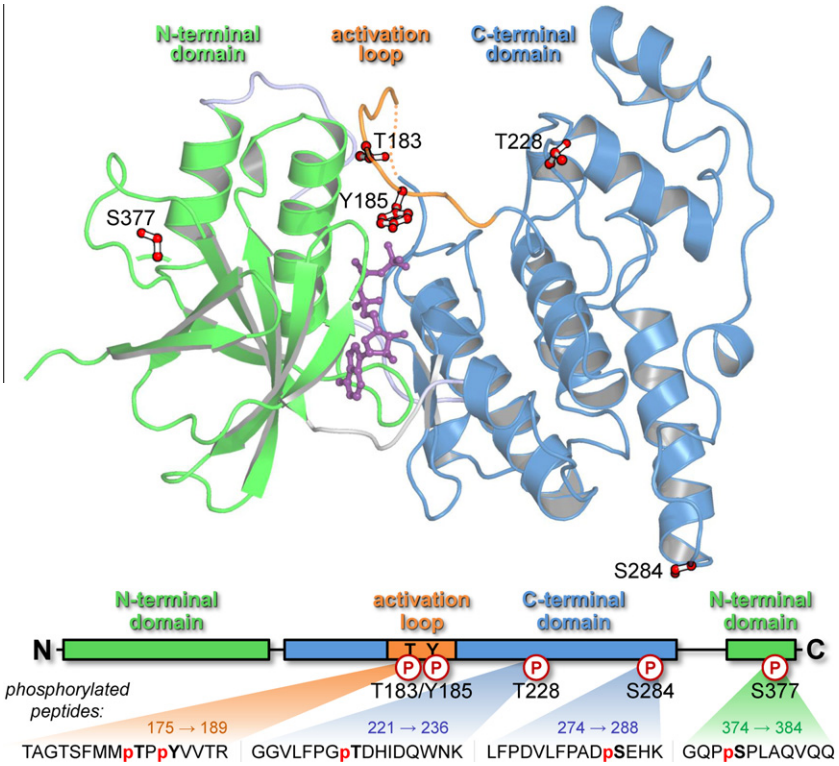

Fig. 2. Mapping JNK1β1 residues that are phosphorylated both during expression in *E. coli* and *in vitro* by active MKK4. The tryptic phospho-peptides identified and the location of the canonical (T183/Y185) and novel (T228/S184/S377) phosphorylated residues are shown. As the C-terminal residues L364-Q384 were not resolved in the crystal structure, an additional serine has been used to indicate the position of S377. The protein structure was rendered using PyMOL™ v. 0.99 (DeLano Scientific, San Carlos, CA) using the PDB code 3ELJ [18].

relative activity of the kinase if they do, we monitored the rate at which p*JNK1β1 produces an electrophoretic shift in ATF2 observed on a Tricine–SDS-PAGE gel when it is singularly and doubly phosphorylated (the first report of its kind) (Fig. 3). This novel approach to an electrophoretic mobility shift assay revealed that p*JNK1β1 was in fact active and able to phosphorylate ATF2, but at a relatively slow rate (panel A, Fig. 3). JNK1β1 is thus activated in *E. coli* by, not unexpectedly, phosphorylation of its T183/Y185 (and possibly the other) residues, yet the small degree at which it had become modified in the bacterium (Fig. 1) can account for the low activity displayed.

Considering recent findings, only a few possibilities exist why JNK1β1 may become phosphorylated during bacterial overexpression (Supplementary Fig. S6, and discussed briefly in [8]). It is

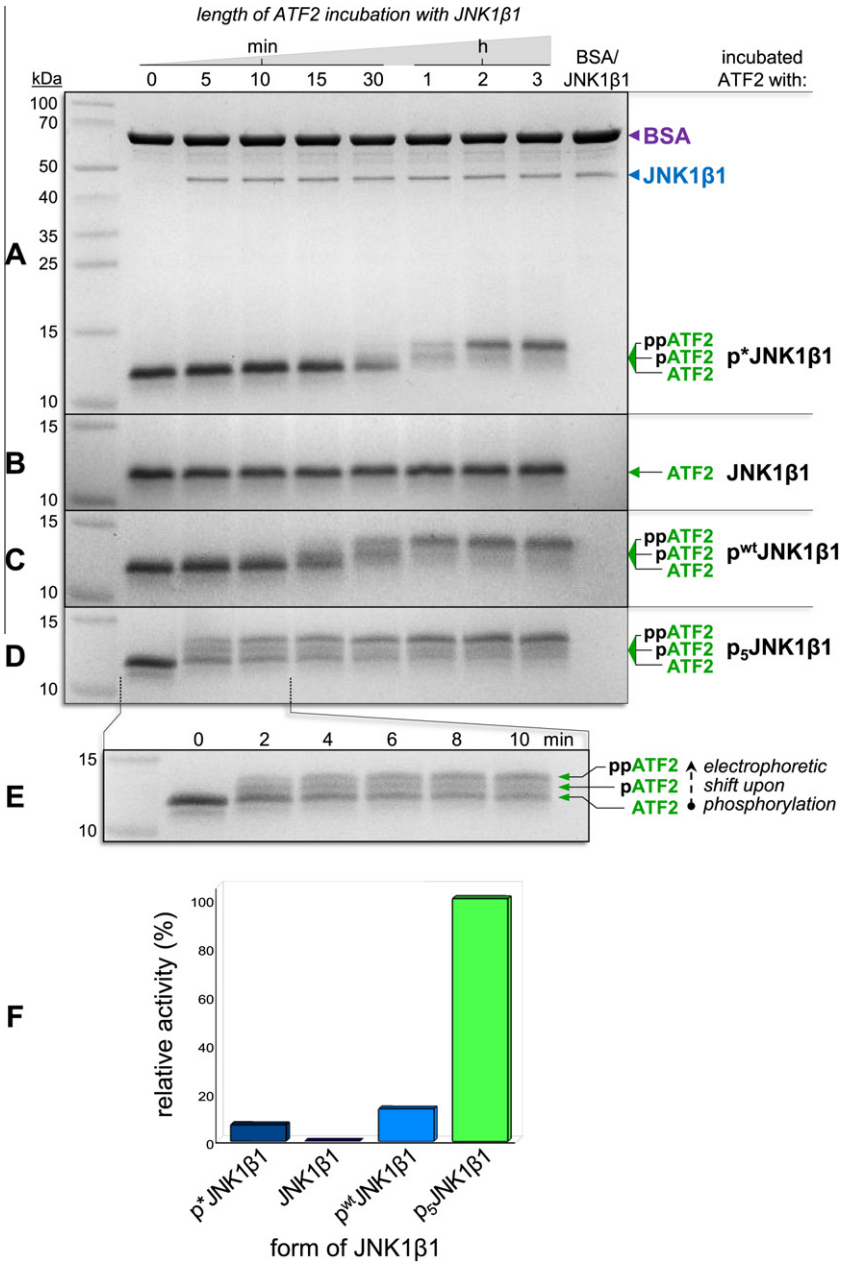

Fig. 3. Relative catalytic activities of the JNK1β1 phospho-forms. (A–E) The rate at which each JNK1β1 phospho-form induces an electrophoretic shift in ATF2(17–96), observed on a 16% Tris–Tricine gel, when it is singularly and doubly phosphorylated at T69/T71 (as confirmed in [8]) was used as an indication of their catalytic activities. (A) The slow phosphorylation of ATF2 by *E. coli* purified p*JNK1β1, and the lack thereof by (B) dephosphorylated, inactive JNK1β1. (C) The slow rate of ATF2 phosphorylation by p^{wt}JNK1β1 partially phosphorylated by untreated MKK4. (D and E) Efficient ATF2 phosphorylation by p_5JNK1β1 phosphorylated by ppMKK4. 1 μM of each JNK1β1 was incubated with 30 μM ATF2, 0.2 mg/ml BSA, 10 mM $MgCl_2$, and 1 mM ATP for 3 h at 25 °C. (F) Graphical comparison of the relative activities determined in A–E, each expressed as a percentage of the activity of p_5JNK1β1.

recognised that kinases may undergo phosphorylation/autophosphorylation during heterologous protein expression. Shrestha et al. [11] has suggested that as the cell is flooded with recombinant protein during overexpression, native kinases are capable of phosphorylating the newly-produced kinase polypeptides during co- or post-translational folding. This would allow phosphate groups to be introduced at both canonical and/or non-standard sites that may or may not be surface exposed. Their study analysed the conditions under which human kinases exhibit autophosphorylation during overexpression, and demonstrated indirectly that altering the kinetics of protein production and folding by adjusting the induction temperature and/or inducer concentration, influences the degree of phosphorylation.

Since native, fully folded JNK1 is unable to undergo post-translational autophosphorylation both *in vivo* and *in vitro* [8,12], the *E. coli* phosphorylation of JNK1β1 described here may be due to this "co-folding autophosphorylation" phenomenon (Fig. S6). As the phosphorylation of non-codon harmonised JNK1 during its overexpression has not been observed elsewhere or reported before, it is possible that the unique yet natural kinetics of translation and folding of our JNK1β1 provided by codon usage harmonisation [8], may allow phosphorylation to occur during co-translational folding. Since codon harmonisation is the only noteworthy difference in our expression system when compared to those used elsewhere for JNK1, these other systems act as numerous controls that highlight the absence of phosphorylation of non-codon

harmonised JNK1 when expressed in *E. coli*. It is therefore fair to propose that the translation kinetics of JNK1β1 influence its ability to be phosphorylated during its overexpression, as implied by Shrestha et al. [11].

We also cannot exclude the idea that unidentified endogenous *E. coli* S/T/Y kinases may be responsible for the phosphorylation of JNK1β1 during expression, as initially suggested by Yang and Lui [13]. It has been found recently that, while few S/T/Y kinases have been directly identified in prokaryotes, at least 80 bacterial proteins are naturally phosphorylated at S/T/Y residues [14]. Considering the recently realised existence of these eukaryotic-like bacterial kinases, it is becoming increasingly unwise to assume that recombinant eukaryotic proteins will purify in a homogenous, non-phosphorylated form [14,15]. It is therefore a possibility that *E. coli* kinases may recognise JNK1β1 in a manner similar to that of its natural activator, MKK4, thereby catalysing its phosphorylation (Fig. S6). As this has not been previously observed for JNK, it is likely that this too occurs during co-translational folding, since the natural kinetics of translation of JNK1β1 imparted by our codon harmonisation may now permit bacterial kinases to interact with phosphorylatable motifs during the translational folding of JNK1β1 (Fig. S6).

Interestingly, only the soluble cell fraction contained phosphorylated JNK1β1 (Fig. S2). It is therefore conceivable too that either co-translational phosphorylation of JNK1β1 promotes its proper native folding, or perhaps only the partially folded JNK1β1 polypeptides following the native folding pathway are able to be phosphorylated during translation.

3.2. Dephosphorylation and deactivation of E. coli purified JNK1β1

We initially dephosphorylated p*JNK1β1 using shrimp alkaline phosphatase (SAP) and monitored the reaction by Western blotting [8], yet to verify that the phosphate groups that had been added during expression had all been removed, we also performed intact LC–MS on the dephosphorylated JNK1β1. It was important to establish that this could be achieved because, due to the inaccessibility of some of the phospho-residues upon final folding of the protein, the incorporated phosphate groups may not be able to be removed by subsequent treatment with phosphatases [16]. The LC–MS analysis revealed that after incubation of p*JNK1β1 with SAP for 5 h, the kinase becomes completely dephosphorylated as the additional peaks situated at multiples of 80 Da above the mass of non-phosphorylated JNK1β1 seen in untreated kinase were no longer detected (profile 2, Fig. 1). Testing the catalytic activity of the kinase also revealed that dephosphorylation completely deactivates the kinase, as all of it activity toward ATF2 was eliminated (panel B, Fig. 3). The phosphate groups introduced during expression were thus all available and susceptible to SAP dephosphorylation, consistent with the observed external accessibility of the phospho-residues (Fig. 2).

3.3. JNK1β1 phosphorylation by MKK4 in vitro

Upon acquiring non-phosphorylated JNK1β1, we sought to generate phosphorylated, fully active JNK1β1 by reconstituting the JNK MAPK cascade *in vitro*.

To first control for the effect of unmodified, wild-type MKK4 (that has not encountered MEKK-C), we first incubated untreated MKK4 with dephosphorylated JNK1β1, and evaluated the change in its state of phosphorylation by intact LC–MS. Interestingly, JNK1β1 is able to be re-phosphorylated to a small degree by untreated MKK4, producing small populations of singly (18%) and doubly (12%) phosphorylated JNK1β1 (p^{wt}JNK1β1, profile 3, Fig. 1). This suggests that MKK4, when recombinantly expressed in *E. coli*, also becomes partially phosphorylated. This finding reiterates the value of determining if heterologously expressed proteins have already undergone co- or post-translational modifications before performing downstream analyses that may be affected by their presence. p^{wt}JNK1β1 is also partially active and can phosphorylate ATF2 (panel C, Fig. 3), yet also at a slow rate due to its ineffective activation and the consequential low population of phosphorylated kinase (profile 3, Fig. 1).

Efficient phospho-activation of JNK1β1 was then achieved by incubating active MKK4 (ppMKK4; previously phosphorylated by MEKK-C), with dephosphorylated JNK1β1 for $\geqslant$3 h at a 10:1 JNK1β1:ppMKK4 molar ratio. Intact LC–MS indicated that JNK1β1 is phosphorylated *in vitro* at five sites by ppMKK4, and to a sufficient efficiency that only a small population of JNK1β1 was not modified at all (p_5JNK1β1, profile 4, Fig. 1). Significant levels of the other phospho-forms existed, with the largest populations comprising singularly and doubly phosphorylated JNK1β1 (profile 4, Fig. 1).

Peptide sequencing of a tryptic digest of p_5JNK1β1 illustrated that ppMKK4 not only phosphorylates JNK1β1 efficiently and expectedly at its canonical T183/Y185 residues *in vitro*, but also unequivocally at T228, S284, and S377 (Fig. 2, and see Fig. S5 for the MS/MS product ion spectra of the resulting phospho-peptides). Curiously, these are the identical sites at which JNK1β1 became phosphorylated during expression in *E. coli*, albeit to a significantly higher degree (Figs. 1 and 2). The initial complete dephosphorylation of the residues phosphorylated during expression followed by their subsequent *in vitro* re-phosphorylation by ppMKK4, suggests that the *E. coli* phospho-activation of JNK1β1 is more a systematic than random event. In other words, the phosphorylation of JNK1β1 in *E. coli* appears to mimic the events that occur in its natural host environment, since the canonical and additional sites were phosphorylated both endogenously in *E. coli* and exogenously by reconstituting the JNK MAPK pathway.

While S377 has been identified previously as a JNK1β1 phospho-site in MS-based proteomics analysis of the human kinome (Table S1), its physiological relevance or the regulatory kinase/s that catalyse its phosphorylation has not been characterised. T228 and S284 are newly identified JNK1β1 phospho-sites, and novel residues known to be phosphorylated by MKK4. S377 and S284 are fully conserved across the JNK splice variants, while T228 is conserved within the β variants of JNK1 and the α variants of JNK2 (Fig. S1). Phosphorylation of S377 and S284 may therefore represent a general mechanism for JNK regulation, whereas T228 phosphorylation may allow differential regulation of the relevant JNK variants to induce their divergent and partially contradictory cellular actions.

A possible explanation for the phosphorylation of these residues is that, once activated by the ppMKK4-dependent phosphorylation of T183/Y185, JNK1β1 then phosphorylates itself at T228/S284/S377. While this may be possible for S377, neither T228 nor S284 is followed by a proline, a requirement for JNK to phosphorylate other proteins [4]. This strongly suggests that at least T228 and S284 are phosphorylated by ppMKK4.

To begin to understand the biological role of the three additional JNK1β1 phospho-sites, we intend to determine the conditions under which pT228, pS284 and pS377 naturally occur in human cells (e.g. 293T cells), and how they affect normal cellular functioning. We also intend to fully elucidate the structural and thus functional relevance of T228/S284/S377 phosphorylation, yet examining the relative activity of p_5JNK1β1 indicates that these modifications, in combination, do not have an overall or net inhibitory effect on the kinase. It is clear that p_5JNK1β1 is highly active and phosphorylates ATF2 efficiently (Fig. 3), emphasising the effectiveness at which ppMKK4 generates large quantities of phosphorylated, active JNK1β1 (profile 4, Fig. 1). We have also previously determined the specific activity of p_5JNK1β1, which was measured

at a high 3.3 ± 0.4 μmol of ATF2/min/mg of kinase [8]. Although the relevance of the three additional phospho-sites may not be known at present, it is possible that in addition to the effect that they may have on the activity of JNK1β1, they may play other roles that could include the regulation of; the interaction of JNK1β1 with its >50 binding partners [4], its translocation between different cellular compartments, the rate at which T183/Y185 are dephosphorylated, or the stability of the kinase.

Furthermore, T228 and S284 neighbour the N-capping motifs of the α-helices comprising N229-L241 and E285-M301, respectively (Fig. 2). Helix-capping motifs contribute to the conformational stability of both the α-helix and the overall protein in which they are situated, and as such the capacity for phosphorylation to regulate the function of proteins by modulating the stability of their α-helices is well known (e.g. see [17]). The locations of T228 and S284 therefore allow this mechanism to be a promising means by which their phosphorylation may affect JNK1β1 functioning.

Evaluating the propensity that each JNK1β1 phospho-site has to be phosphorylated *in vitro* by ppMKK4 by comparing the relative abundance of the phosphorylated and non-phosphorylated populations of each precursor p_5JNK1β1 phospho-peptide (see Supplementary material), the order of their phosphorylation propensity was determined to be: T183/Y185 > S377 > T228 ⩾ S284. As T183 and Y185 have, unsurprisingly, the greatest predisposition to be phosphorylated, p_5JNK1β1 likely comprises approximately 60% kinase phosphorylated on at least T183 and Y185.

The relatively low percentage of JNK1β1 phosphorylated at either pT228 and/or pS284 (each comprising ±10% of the total phosphorylated kinase population; Table S2) may account for their previous non-detection in mammalian cells, yet the power and sensitivity of single-protein mass spectrometry (as performed here) confirm that these phospho-sites are not false positives.

Our studies provide important insights into the fate of JNK1β1 overexpressed in *E. coli*, raising interesting new questions of the possibility that endogenous *E. coli* kinases may be involved in the activation of heterologously expressed MAPKs. This points at the prospect that prokaryotic kinases may recognise eukaryotic phosphorylation motifs, or that there may be degree of evolutionary relatedness between prokaryotes and eukaryotes. We are however not able to affirmatively conclude that JNK1β1 is subject to phosphorylative activation by endogenous bacterial kinases because this study was not designed to prove this hypothesis. Nevertheless, future research could be aimed at exploring and identifying bacterial kinases possibly capable of phosphorylating JNK1β1 and other eukaryotic proteins that undergo post-translational modification. The concept of using a codon-harmonised JNK1β1 gene to improve or naturalise its transitional kinetics to prime the protein for co- or post-translational modification, catalysed either by itself or endogenous host proteins, also has significant implications for all heterologous protein expression, highlighting the value of its further investigation. Furthermore, the functional and biochemical relevance of the novel JNK1β1 phosphorylation sites we have identified could also give important insights into the molecular mechanisms by which JNK1β1 is regulated, as well as shed light on the differential regulation and hence functional relevance of the different JNK splice variants.

Acknowledgments

This work was supported by the University of the Witwatersrand, South African National Research Foundation (Grants: 60810, 65510 and 68898 to H.W.D) and South African Research Chairs Initiative of the Department of Science and Technology and National Research Foundation (Grant: 64788 to H.W.D).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.018.

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