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A scanning peptide array approach uncovers association sites within the JNK/βarrestin signalling complex

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ABSTRACT

βarrestins are molecular scaffolds that can bring together three-component mitogen-activated protein kinase signalling modules to promote signal compartmentalisation. We use peptide array technology to define novel interfaces between components within the c-Jun N-terminal kinase (JNK)/βarrestin signalling complex. We show that βarrestin 1 and βarrestin 2 associate with JNK3 via the kinase N-terminal domain in a region that, surprisingly, does not harbour a known 'common docking' motif. In the N-domain and C-terminus of βarrestin 1 and βarrestin 2 we identify two novel apoptosis signal-regulating kinase 1 binding sites and in the N-domain of the βarrestin 1 and βarrestin 2 we identify a novel MKK4 docking site.

Structured summary:
MINT-7263196, MINT-7263175: *Arrestin beta-2* (uniprotkb:P32121) *binds* (MI:0407) to *ASK1* (uniprotkb:Q99683) by *peptide array* (MI:0081)
MINT-7263136: *JNK3* (uniprotkb:P53779) *binds* (MI:0407) to *Arrestin beta-1* (uniprotkb:P49407) by *peptide array* (MI:0081)
MINT-7263161: *JNK3* (uniprotkb:P53779) *binds* (MI:0407) to *Arrestin beta-2* (uniprotkb:P32121) by *peptide array* (MI:0081)
MINT-7263304: *Arrestin beta-1* (uniprotkb:P49407) *physically interacts* (MI:0915) with *ASK1* (uniprotkb:Q99683) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7263286: *Arrestin beta-2* (uniprotkb:P32121) *binds* (MI:0407) to *MKK4* (uniprotkb:P45985) by *peptide array* (MI:0081)
MINT-7263231, MINT-7263254: *Arrestin beta-1* (uniprotkb:P49407) *binds* (MI:0407) to *ASK1* (uniprotkb:Q99683) by *peptide array* (MI:0081)
MINT-7263269: *Arrestin beta-1* (uniprotkb:P49407) *binds* (MI:0407) to *MKK4* (uniprotkb:P45985) by *peptide array* (MI:0081)

1. Introduction

Mitogen-activated protein kinase (MAPK) signalling cascades of the ERK (extracellular-signal regulated kinase), p38 and JNK pathways (c-Jun N-terminal kinase) are activated via cell surface receptors in response to a variety of physical and chemical stimuli [1]. In each case, signals are relayed in a three-kinase sequence, going from MAPK kinase kinase through MAPK kinase to MAPK. Activated MAPKs have the ability to elicit a myriad of cellular responses by phosphorylating a large number of target proteins, which include other kinases, enzymes and transcription factors. Each individual MAPK cascade is activated by a unique set of stimuli, though it is also known that distinct MAPK modules can be triggered in parallel by a common initiating event [2]. Despite the plethora of possible stimuli and small number of MAPK signalling cascades, the MAPK signalling networks are known to operate in a highly regulated manner with specificity of action being maintained by a family of unconnected scaffold proteins [3]. Such signalling scaffold proteins act to bring the components of each pathway together, allowing physical association of pathway members to promote rapid transmission of the signal coupled with spatial sequestration, which confers compartmentalisation upon signal output [4].

Abbreviations: MAPK, Map kinase; JNK, c-Jun N-terminal kinase; ASK1, apoptosis signal-regulating kinase 1; MKK4, MAPK kinase 4; CD site, common docking site

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βarrestins are multi-functional signal scaffold proteins that are known to sequester the three tier kinase modules from the ERK, p38 and JNK signalling cascades [5]. JNK3 was originally shown to associate with βarrestin in a yeast two-hybrid screen with further experimentation showing that βarrestin could scaffold not only the MAPK, JNK but also the MAPK kinase, MAPK kinase 4 (MKK4) and the MAPK kinase kinase, ASK1 (apoptosis signal-regulating kinase 1) [6,7]. More recent studies into the mechanism of assembly of MAPK modules by βarrestins have suggested that all four mammalian isoforms of βarrestin are able to bind the three components of the JNK module (JNK, MKK4 and ASK1) with comparable affinity and that all of these kinases associate with both the N- and C-domains of the βarrestin proteins [8,9]. Traditionally, analyses to determine interaction sites are both time-consuming and expensive, involving truncation, domain swapping and site-specific mutagenesis studies. Truncation approaches together with a limited number of mutagenesis analyses have been used to roughly define the molecular interaction sites within the βarrestin/JNK module complex [6,8–11]. Use of such approaches has, however, yielded little information about key sites involved in the formation of the βarrestin/JNK complex and hence there is a paucity of information on the residues involved.

Recently, peptide array technology has proved to be a novel, rapid and powerful tool for studying protein–protein interactions in a number of systems (see e.g. [12,13]) and has been verified for the definition of optimal docking sequence motifs with members of MAPK cascades [14]. The technique has also been utilised to accurately predict the binding sites on βarrestin for a number of interacting partners including PDE4D5 [15], ERK [16] and MEK [17]. Here we have employed this technique to further delineate the binding site for βarrestin on JNK and to uncover potential sites that allow βarrestin to scaffold both ASK1 and MKK4.

2. Materials and methods

Peptide libraries were produced by automatic SPOT synthesis and synthesised on continuous cellulose membrane supports on Whatman 50 cellulose membranes using Fmoc-chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany) as previously described [18]. The interaction of spot-immobilised peptides with purified, recombinant GST and βarrestin 1–GST, βarrestin 2–GST fusion proteins, MKK4 and ASK1 was determined by overlaying the cellulose membranes with 10 μg/ml protein as previously described [19]. The purification of GST, βarrestin 1–GST and βarrestin 2–GST were as previously described [19]. Pure MKK4 (codes 14–377) and active ASK1–GST (codes 14–606) and antibodies against MKK4 (04-382) were purchased from Upstate (Millipore), UK. Anti-GST monoclonal antibody (SC-53909) was purchased from Santa Cruz. Densitometric analysis was done using Quantity One software (Biorad). The immunoprecipitation of FLAG-βarrestin 1 and subsequent blotting for the association of ASK1 was done as previously described for MEK1 [17].

3. Results

3.1. Elucidation of βarrestin-binding site on JNK3

In this study, we used peptide array analysis to probe various interactions within the JNK/βarrestin system. To do this we exploited peptide array technology to synthesise libraries of overlapping 25-mer peptides, each shifted by five amino acids, spanning the complete sequence of JNK3, βarrestin 1 and βarrestin 2. Recent studies investigating the association of the ASK1–MKK4–JNK3 module with βarrestins have indicated that the N-terminus of JNK3 harbours the site of association with βarrestins and that both βarrestin 1 and βarrestin 2 appear to bind JNK3 with similar ability [8,11]. In agreement with such findings, purified βarrestin 1–GST and βarrestin 2–GST, but not GST, bound similarly to 25-mer peptides representing a full length JNK3 peptide array (Fig. 1A), identifying an interaction site within the JNK3 N-terminal region that spanned amino acids 6–45. In order to define further the amino acids that potentially allow binding of JNK3 to βarrestin 1 and βarrestin 2, we screened a family of 22-mer peptides whose sequence contained L^3–F^{24} of JNK3. The 22 progeny of this peptide each contained a sequential single substitution of alanine for successive amino acids to form an alanine-scanning array that was once again probed with βarrestin 1 and βarrestin 2–GST. When this region was subjected to such binding analysis, it became apparent that βarrestin 1 and βarrestin 2–GST binding profiles were similar. Notably, alanine substitution of F^5 compromised βarrestin 1 and βarrestin 2 binding (Fig. 1B) and the run of residues between P^{12} and G^{23}, save D^{15} and A^{19}, more specifically, compromised βarrestin 2 binding (Fig. 1B). Gratifyingly, such a result is in agreement with previous truncation and site-directed mutagenesis studies [11], which have shown the JNK N-terminal and, in particular, amino acids between C^9 and I^{18} to be important for the association of βarrestin. However, our approach extends appreciation of the interaction surface on JNK, in that residues F^5 and C^{21}–F^{24} also appear have a role to play in the context of βarrestin 1 and βarrestin 2/JNK interaction, while D^{15} likely is unimportant.

3.2. ASK1 binds to N- and C-domains of βarrestin 1 and βarrestin 2

To discover elements within ASK1 and βarrestin 2 that mediate their association, a recent study utilised over-expression of individual ASK1 domains to demonstrate that only constructs containing a combination of the kinase and C-terminal domains, but not either the isolated N- or C-terminal domains of ASK1 alone, could bind βarrestin 2 [8]. These data strongly suggested that the kinase domain of ASK1 contained the crucial βarrestin-binding determinants. The same study, however, reported that both the N- and C-domains of βarrestin 2 could associate independently with ASK1 [8], though the precise sites of interaction were not elucidated. Here, we show that, in agreement with this work, a GST fusion containing the kinase domain of ASK1 (amino acids 649–946) bound specifically to two cognate regions on βarrestin 1 and βarrestin 2 peptide arrays. One region was located in the N-domain, spanning amino acids 6–35 in βarrestin 1 (Fig. 2A, Spots 2 and 3) and amino acids 6–45 in βarrestin 2 (Fig. 2C, Spots 2, 4 and 5) and one was in the C-domain, spanning βarrestin 1 amino acids 376–410 (Fig. 2B, Spots 76, 77 and 78) and βarrestin 2 residues 381–405 (Fig. 2D, Spot 77). Purified GST failed to associate with any spot within the βarrestin1 and βarrestin 2 arrays (Fig. 2).

To gain further insight into the amino acids from the βarrestin 1 and 2 N-domains that bind specifically to the ASK1 kinase domain, we undertook alanine-scanning analysis of these regions (Fig. 2E–H). Doing this, however, we saw no obvious single alanine substitution causing loss of binding of the ASK1 kinase domain to βarrestin 1 (Fig. 2E). Intriguingly, however, several of the alanine substitutions (V^{28}, H^{30} and G^{39}) resulted in enhanced association of the ASK1 kinase domain, compared with the unsubstituted control, using this peptide from the βarrestin 1 N-domain (Fig. 2E). Such data further indicate that ASK1 binds this peptide, is sensitive to changes in its sequence and suggests that multiple substitutions of amino acids within it may be necessary to compromise ASK1 kinase domain binding. Indeed, in this regard, we determined that a double substitution of D^{26}:D^{29} ablated binding in both a peptide array (Fig. 2E) and in vivo when a D^{26}A:D^{29}A βarrestin 1 double mutant was overexpressed in HEK cells (Fig. 3D). Interestingly, these amino acids have been indicated in the binding of another kinase (MEK1) to βarrestin 1 [17]. The