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Does MK5 reconcile classical and atypical MAP kinases?

Maria Perander[1], Stephen M. Keyse[2], Ole-Morten Seternes[1]

[1]Department of Pharmacology, Institute of Medical Biology, University of Tromso, N-9037 Tromso, Norway, [2]Cancer Research UK Stress Response Laboratory, Biomedical Research Centre, Level 5, Ninewells Hospital and Medical School, Dundee DD1 9SY, United Kingdom

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1. ABSTRACT

MAP kinase-activated protein kinase 5 (MK5) was originally described as a protein kinase activated downstream of the p38 MAP kinase and is also named p38-regulated/activated protein kinase (PRAK). However, while MK5 is most similar in sequence to the two p38 regulated MAPKAP kinases MK2 and MK3, recent data has shown that in contrast to these enzymes MK5 is not activated in response to either cellular stress or pro-inflammatory cytokines. This lack of response to stimuli which cause robust activation of p38 MAP kinase *in vivo* is supported by data obtained using transgenic mice lacking MK5. Unlike animals lacking MK2 and MK3, MK5 null mice respond normally to endotoxic shock and display an unchanged pattern of cytokine expression in response to LPS. Clues as to the physiological function of MK5 have come from the recent observation that MK5 is uniquely regulated and activated following complex formation with the atypical MAP kinases ERK3 and ERK4. Thus, it is possible that MK5 is unique amongst the MAPKAP kinases in being regulated downstream of signaling pathways other than the classical MAP kinases p38 and ERK1/2.

2. THE DISCOVERY OF MK5 AS A P38 REGULATED AND ACTIVATED KINASE

Mitogen-activated protein kinase (MAPK)-activated protein kinase 5 (MAPKAP kinase-5 or MK5) was first identified in two independent searches of the human and mouse expressed sequence tag (EST) databases for proteins with sequence homology to the prototypic MAPKAP kinase MK2 (1, 2). Both groups subsequently showed that MK5 could be phosphorylated and activated by the stress-activated p38 MAP kinase, leading New et al. to name this kinase as p38 regulated and activated kinase or PRAK. More recently, MK5 has also been shown to interact with the atypical MAP kinases ERK3 and ERK4 and this also results in phosphorylation and activation of MK5 (3-6). The amino acid sequence of MK5 is most closely related to MK2 (32.8%) and MK3 (33.1%), but MK5 is more distantly related to these kinases than they are to each other (Figure 1A) (1). An MK5 gene does not appear to be present in either *C. elegans* or *Drosophila*, but orthologues are found in most vertebrates.

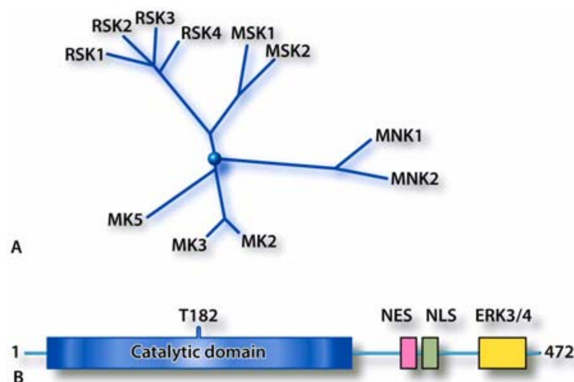

Figure 1 Sequence relationship between different MAPKAP kinases and structure of the MK5 protein A Phylogenetic tree of the MAPKAP kinase family members. This includes 4 ribosomal-S6 kinases (RSK1-4), 2 mitogen and stress-activated protein kinases (MSK1 and 2), 2 MAPK interacting kinases (MNK1 and 2) and finally 3 MAPK activated protein kinases (MK2, 3 and 5). B. Schematic primary structure of MK5. The phospho-acceptor site (T182) in the activation loop is shown with numbering based on the human sequence. The Nuclear Export Sequence (NES), the Nuclear Localization Sequence (NLS) and the ERK3 and ERK4 interaction domain (ERK3/4) are shown.

3. STRUCTURE AND EXPRESSION

The human MK5 gene encodes two differentially spliced transcripts giving rise to proteins of 471 amino acids and 473 amino acids respectively. The two isoforms differ such that only two additional amino acids are found within the C-terminal extension of MK5 of transcript variant 2 when compared to transcript variant 1. In mice, five MK5 isoforms have been detected, which represent various combinations of the two amino acid changes in the C-terminal domain found in humans with deletion of the N-terminal portion of the MK5 catalytic domain (7). The functional significance of these different splice variants is as yet unknown. MK5 seems to be expressed almost ubiquitously having been detected in most tissues and cell lines that have been analysed. As found in all other MAPK-activated protein kinases, MK5 contains a conserved LXTP site in the T-loop where the Threonine is the single phospho-acceptor site (1). MK5 also contains a functional nuclear export sequence (NES) and a nuclear localisation sequence (NLS) within the C-terminal domain (8, 9). The NLS overlaps with a MAPK docking site (8-10). In addition, MK5 possesses a 100 amino acid extension C-terminal to the NLS, which is not found in either MK2 or MK3. This portion of MK5 contains sequences that are required for interaction with the atypical MAP kinases ERK3 and ERK4 (Figure 1B).

4. REGULATION OF ACTIVITY

4.1 Is MK5 regulated by the p38 MAPK pathway *in vivo*?

Both ERK2 and p38 α/β are able to activate MK5 *in vitro* (1, 2). Analysis of the protein sequence of MK5 revealed the presence of five Serine/Threonine-Proline motifs that are potential sites for MAPK-dependent phophorylation. However, phospho-peptide mapping of MK5 phosphorylated *in vitro* by p38 demonstrated that T182 within the conserved LXTP motif of the activation loop in kinase domain VIII was the only phosphorylated residue. Furthermore, a mutant MK5 in which T182 was substituted by alanine was no longer activated by MAP kinase, demonstrating that MK5 carries the same phospho-acceptor site conserved in the T-loop of all other MAPKAP kinases (1). However, MK5 is unusual in requiring only this single phosphorylation event for activation, as MK2 and MK3 both require at least one additional residue to be modified. Comparison of the MK5 sequence with that of MK2 and 3 revealed that these additional regulatory MAPK phosphorylation sites are not found in MK5. Finally, New and coworkers systematically mutated the remaining four potential MAPK phosphorylation sites to Ala (1). All of these proteins were activated *in vitro* to the same extent as wild-type MK5 by p38, confirming that T182 is indeed the only regulatory phosphorylation site within MK5.

Despite the fact that when over-expressed in cells MK5 can clearly be activated by p38 MAPK in a similar way to the related MAPKAP kinase MK2 (1, 8, 9) doubts began to surface as to whether or not MK5 was a *bona-fide* target of the p38 MAP kinase pathway *in vivo*. The original publication by New *et al.* suggested that the small heat shock protein Hsp27 was a physiological target for MK5 (1). However, when knockout mice lacking the MK2 gene were characterised there was little or no residual p38-dependent Hsp27 kinase activity in cells and tissues derived from these animals (11). More recently, the murine gene encoding MK5 itself has been deleted. Unlike MK2 knockout mice, these animals display a normal profile of LPS-induced cytokine production, show no increased resistance to endotoxic shock and a detailed analysis of protein-protein interactions between endogenous MK5 and p38 failed to find any evidence for complex formation (11, 12). Furthermore, while deletion of MK2 caused a marked destabilisation of p38 MAPK due to loss of its ability to act as a chaperone for this MAPK, loss of MK5 had no effect on the stability of p38 (11). Finally, no significant activation of endogenous MK5 could be detected in response to either cellular stress or inflammatory cytokines, despite the concomitant activation of both p38 MAPK and MK2 (11).

4.2 The activation of MK5 can be mediated by interactions with ERK3 and ERK4

The recent finding that MK5 forms specific complexes with the atypical MAPKs ERK3 and ERK4 strongly indicates that MK5 may mediate functions that are completely independent of the p38 MAPK signalling pathway. Co-expression of either ERK3 or ERK4 with MK5 leads to increased phosphorylation of T182 with concomitant activation of MK5. While there is some controversy regarding the requirement for kinase activity of ERK3 for this activation (3, 4), the activation of MK5 by ERK4 is clearly dependent on the kinase activity of ERK4 (5, 6). In support of a physiological role for ERK3 in MK5 activation, the activity of MK5 is significantly

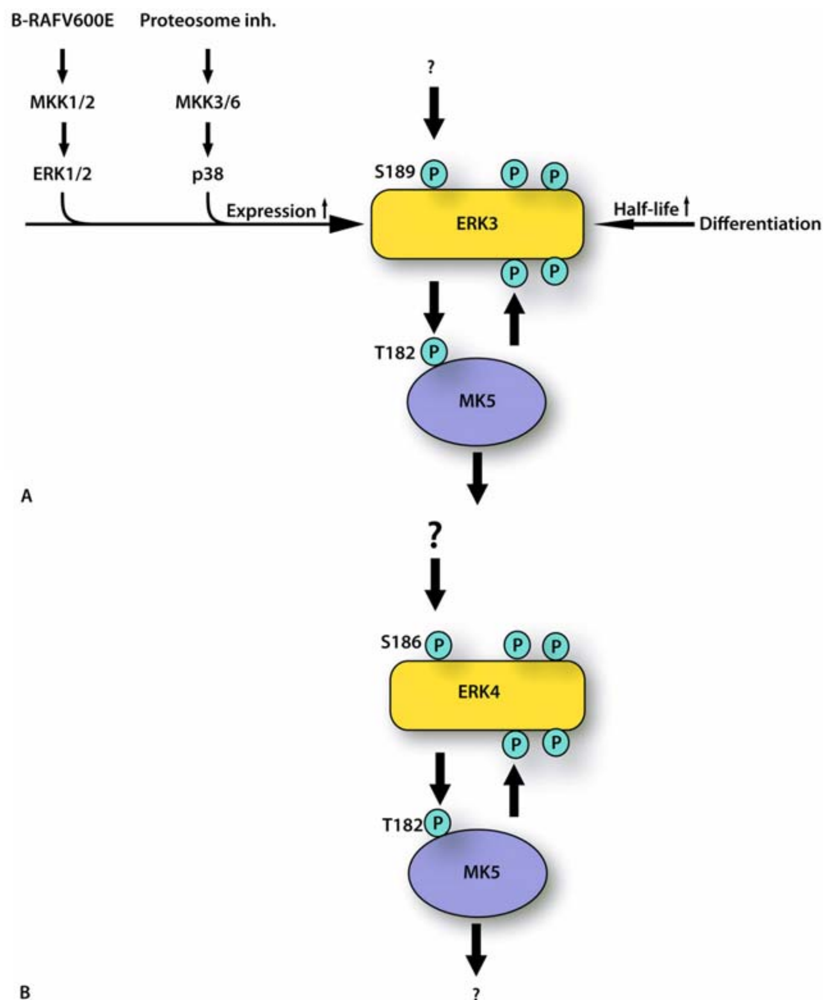

Figure 2. Both ERK3 and ERK4 can activate MK5. A ERK3 binds to MK5 and activates MK5 through phosphorylation of MK5 on T182. Once activated, MK5 then phosphorylates its binding partner ERK3. So far no agonist has been shown to stimulate ERK3 activity. ERK3 is a protein with a short-half life and its activity is suggested to be regulated at the level of cellular abundance. ERK3 can be regulated at the level of transcription by activation of the MEK-ERK signalling pathway via oncogenic B-RAF and also via the activation of the p38 signalling pathway by proteosome inhibitors (14, 27). In addition, ERK3 is also regulated at the level of protein stability during cell differentiation (13). B Like ERK3, ERK4 also binds to MK5 and activates MK5 through phosphorylation of T182. Once activated, MK5 also phosphorylates ERK4. In contrast to ERK3, ERK4 is a relatively stable protein and nothing is known about the regulation of ERK4 activity, expression and protein turnover.

reduced in mouse embryo fibroblasts (MEFs) derived from ERK3 knock-out animals when compared to levels seen in MEFs derived from wild-type mice (3, 6). Conversely, siRNA mediated knock down of both ERK3 and ERK4 in HeLa cells causes a synergistic reduction in endogenous MK5 activity, demonstrating a physiological role for both of these kinases in regulating MK5 (3-6).

ERK3 and ERK4 differ from the classical MAP kinases in several respects. Most strikingly, the highly conserved TXY motif is replaced by SEG and both kinases also contain unique C-terminal extensions. ERK3 is not activated by any known classical MAP kinase stimuli. ERK3 is a highly unstable protein that is subject to ubiquitination and degradation via the 26S proteasome pathway. This appears to be mediated by two destabilisation regions in the N-terminal lobe of the kinase domain (13). It has also been demonstrated that MK5 has an important role in the regulation of the stability of the ERK3 protein. Over-expression of MK5 stabilises over-expressed ERK3 and more importantly, endogenous ERK3 protein levels are severely reduced in cells where MK5 has been knocked down by siRNA and also in embryonic fibroblasts from MK5 null mice (3, 4). Interestingly, upon differentiation of PC12 and C2C12 cells to the neuronal and muscle lineages, respectively, ERK3 is stabilized (13) (Figure 2). The increase in ERK3 protein level in differentiated PC12 cells is accompanied by elevated MK5 activity (4). Recent data has also shown that the expression of ERK3 can be induced at the transcriptional level by oncogenic B-RAF (14). This induction of ERK3 expression by oncogenic B-RAF seems

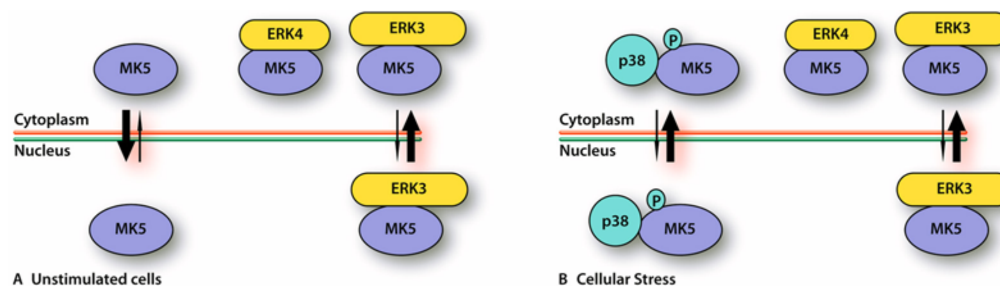

Figure 3. Regulation of MK5s subcellular distribution. A. Over-expressed MK5 is preferentially found in the nucleus of resting cells. However due to the presence of both a functional nuclear export signal and a nuclear localization signal the protein is able to shuttle between the nucleus and cytoplasm. Co-expression with either ERK3 or ERK4 leads to translocation of MK5 to the cytoplasm. The cytoplasmic localization of the ERK3-MK5 complex is dependent on active nuclear export. B. Treatment of cells with either sorbitol or sodium arsenite may lead to nuclear export of MK5 and this is dependent on both p38 MAPK binding and p38 MAPK mediated phosphorylation of MK5 at T182.

to depend on the activities of MEK1/2 and ERK1/2. Whether this induction of ERK3 expression also results in increased MK5 activity is not known. Thus, it is quite clear that ERK3 and ERK4 are important regulators of MK5 activity *in vivo*. However, the physiological circumstances, under which the activation of MK5 by ERK3 and ERK4 occurs, remains enigmatic, particularly as no specific stimuli which lead to the activation of these kinases have yet been identified (Figure 2).

4.3 The interaction of MK5 with ERK3/ERK4 is not dependent on classical MAPK-docking sites

The ability of MAPKAP kinases to interact with and act as substrates for MAPKs *in vivo* is mediated by specific docking domains (D-domains) (15, 16). These D-domains are comprised of short motifs containing basic residues that interact with the MAPK by forming direct contacts with the so-called ”common docking” (CD) domain. This motif, which is comprised of acidic residues, acts as a common binding site for a broad range of MAP kinase interacting proteins including downstream kinases, transcription factors and regulatory phosphatases (17). The D-domains of the various MAPKAP kinases exhibit sequence variations that are important in determining specificity for the upstream MAPK (10). Mutational analysis has shown that exchanging the D-domains of different MAPKAP kinases is sufficient to switch specificity for the upstream activator (18). The D-domain of MK5 can mediate specific interactions with the CD domain of the p38α and β MAPKs and this motif also overlaps with a functional nuclear localization sequence (NLS) suggesting that MAPK binding might also influence the subcellular localization of MK5 (8-10). More recently, MK5 has been demonstrated to bind to the atypical MAP kinases ERK3 and ERK4. Although both of these proteins contain a potential CD-domain, the interaction of ERK3 and ERK4 with MK5 does not require this sequence motif nor is it dependent on the integrity of the D-domain within MK5 that mediates interaction with p38 MAP kinase (4). So far, the exact sequence requirements for the interactions observed between ERK3/4 and MK5 are poorly defined. Deletion of the last 50 amino acids of MK5 is sufficient to disrupt its ability to bind to either ERK3 or ERK4 (4, 6). Conversely, while proteins comprising the first 340 residues of either ERK3 or ERK4 are able to interact with MK5, deletion of a further 10 amino acids from the C-terminus of either protein completely abrogates binding to MK5. Further work is needed in order to fully define the interaction domains of MK5 and ERK3/4 and the determination of crystal structures for these kinases would greatly aid this analysis.

5. SUBCELLULAR DISTRIBUTION

The subcellular localization of endogenous MK5 is somewhat controversial with reports that the endogenous protein is either nuclear or cytoplasmic (8, 9). However, MK5 contains both a functional NLS and a functional NES and although MK5 is found predominantly in the nucleus when expressed in HeLa cells, it can readily shuttle between the nucleus and cytoplasm (8, 9). The latter property may account for the conflicting data, as the subcellular localisation of MK5 could depend on both cell-type and growth conditions. The nuclear localization signal of MK5 overlaps with a functional MAP kinase docking site (8-10), and co-expression of either p38α or p38β interferes with the nuclear import of MK5 resulting in redistribution of a significant fraction of MK5 protein from the nucleus to the cytosol (Figure 3A). Interestingly, the exposure of cells to stressful stimuli also leads to an increase in the nuclear export of MK5 (Figure 3B). This is dependent upon both p38-mediated phosphorylation of T182 in the activation loop and the functional NES within MK5. Cellular stress can also increase the p38-dependent nuclear export of both MK2 and MK3. In the case of MK2, phosphorylation at T334 together with phosphorylation of the activation loop at T222 is essential for stress induced nuclear export. The crystal structure of MK2 shows that T334 lies in the hinge region between the catalytic domain and an auto-inhibitory helix (19). In the inactive state the NES of MK2 is hidden, but phosphorylation of T334 disrupts the interaction between the kinase domain and the C-terminal domain rendering the NES accessible and facilitating nuclear export (20). As mentioned before, MK5 lacks this second phosphorylation site and it would appear that stress-induced nuclear export of MK5 requires binding of p38 and consequent masking of the NLS to promote nuclear export. This may also explain the slower kinetic of stress induced nuclear export observed for MK5 when compared with MK2 (8).

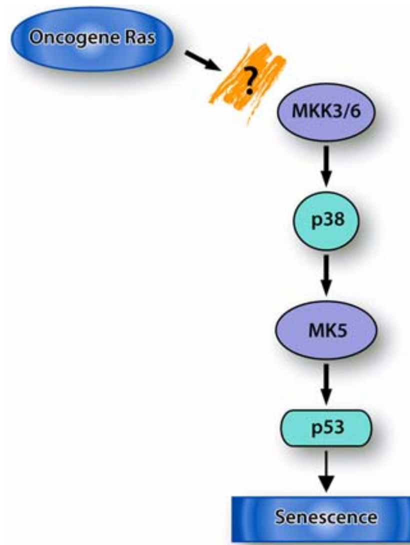

Figure 4. Involvement of MK5 in Ras-induced senescence. Phosphorylation of p53 by MK5 following activation of p38 MAPK by oncogenic Ras is important for Ras-induced senescence

Another mechanism, which may determine the subcellular localisation of MK5, was discovered following the observation that expression of either ERK3 or ERK4 not only leads to activation of MK5 but also the translocation of MK5 from the nucleus to the cytoplasm (Figure 3), (3, 4). The mechanism by which ERK3 and ERK4 mediate nuclear export and cytoplasmic anchoring of MK5 is somewhat different to the process seen following activation of p38 MAPK. Firstly, it does not depend on the kinase activity of either ERK3/4 or MK5 nor does it require phosphorylation of MK5 on T182. Finally, while efficient nuclear export of MK5 following activation of p38 is dependent on the ability of p38 to bind to and mask the NLS within MK5, both ERK3 and ERK4 bind to MK5 through a quite different region of the protein and thus are unlikely to affect NLS function.

Very recently, Protein kinase A (PKA) signalling also been postulated to influence the cellular distribution of MK5 (21). Treatment of cells with forskolin or over-expression of a nuclear-targeted PKA Cα catalytic subunit was shown to induce the transient redistribution of MK5 from nucleus to cytoplasm in rat PC 12 cells (21). Redistribution was not observed on expression of either a kinase dead mutant of MK5 or of MK5 in which the T182 phospho-acceptor site within the kinase activation loop was mutated, indicating a requirement for MK5 activity in this process (21). Further studies will be required in order to ascertain if this interaction is of any physiological relevance.

6. SUBSTRATES

The substrate recognition sequence within MK5 seems to be similar to that of MK2 and MK3 and thus MK5 is also able to phosphorylate HSP25 and HSP27 at the same sites (1, 7). In addition MK5, like MK2, phosphorylate glycogen synthetase, tyrosine hydroxylase (preferentially on S19) and myosin heavy chain *in vitro (1, 2, 22)*. However, despite having a similar substrate specificity to MK2 *in vitro*, the generation of MK5 knock out mice has revealed that MK5 does not contribute to stress dependent HSP27 phosphorylation *in vivo* (11). Instead, recent data has demonstrated that MK5 phosphorylates the atypical MAPKs ERK3 and ERK4 and can also phosphorylate the transcription factor p53 on S37, an activating phosphorylation site, both *in vitro* and *in vivo (*4, 12). The fact that ERK3 and ERK4 are also activators of MK5 is intriguing and suggests that the phosphorylation of these two kinases by MK5 may have important functional consequences.

7. PHYSIOLOGICAL ROLE (S) FOR MK5

Disruption of the MK5 gene in mice of a mixed 129 X C57/B6 genetic background did not give rise to detectable phenotypic changes when these animals were compared with wild-type mice. However, backcrossing of these mice into a C57/B6 genetic background resulted in embryonic lethality with incomplete penetrance (3). The homozygote embryos were underrepresented at least from day E12 and after day E13 autolytic pups could be observed. Interestingly, the time of embryonic death corresponds well with the peak of ERK3 mRNA expression during mouse embryonic development.

Recently, a function for MK5 in protection against tumour development has been inferred from experiments using a distinct line of MK5 null mice (12). These animals, which were on a mixed 129 X C57/B6 genetic background were found to be more susceptible to skin carcinogenesis induced by the mutagen dimethylbenzanthracene (DMBA) when compared with wild type controls. Heterozygous MK5-/+ mice also developed more skin papillomas than

wild type mice after DMBA treatment. More than 90 per cent of skin tumours induced by DMBA contain an activated Ras oncogene, implying that MK5 may play some role in determining the susceptibility of skin cells to ras-induced transformation. Western blot analysis and immunostaining of skin papillomas demonstrated that the expression of senescence-associated proteins including SA-β-gal, p16Ink4a, DcR2 and Dec1 was severely reduced in MK5-deficient tumours compared to tumours from wild type mice. This suggests a role for MK5 in mediating Ras-induced senescence as a means of tumour suppression (Figure 4). Consistent with this, MK5-deficiency also abrogated Ras-induced growth arrest and induction of SA-β-gal, a marker of cell senescence, in murine and human primary fibroblasts. Furthermore, primary mouse embryonic fibroblasts and skin fibroblasts from MK5 -/- mice were much more susceptible to transformation following expression of an oncogenic RasVal12 mutant and Ras-induced senescence could be restored by reintroducing wild type, but not a kinase-dead mutant of MK5. Comparing the activity of MK5 in primary human fibroblasts transduced with RasV12 with control cells showed that MK5 activity was increased in cells over-expressing oncogenic Ras. This activation was inhibited by SB203580, a specific inhibitor of p38α and β indicating that MK5 activation is p38-dependent. In agreement with this, the activity of both p38 and MK5 was enhanced in skin papillomas induced by DMBA in normal mice. Over-expression of oncogenic Ras has previously been shown to activate both MK2 and MK5 in NIH 3T3 cells and co-expression of either MK2 or MK5 suppressed oncogenic Ras-mediated induction of cell proliferation and Ras-dependent gene expression (23). Moreover, the constitutive p38 activator MKK6 also suppressed Ras activity in a p38-dependent manner whereas arsenite, a potent chemical inducer of p38, only inhibited proliferation in a tumor cell line that required Ras activity. The ability of p38α to inhibit Ras-induced transformation has also been demonstrated by Dolado *et al.* (24). In this latter study p38α was not a general inhibitor of oncogenic signaling, but was found to specifically modulate tumourgenesis induced by oncogenes, that lead to the production of elevated levels of reactive oxygen species (ROS) in cells (24). On the basis of these two studies it is clearly important to know the contribution that MK2, which is now well established as a target of the p38 MAPK pathway, in comparison to MK5 actually makes in mediating Ras-induced senescence.

Sun *et al.* suggest that MK5 regulates oncogene-induced senescence by a mechanism involving the p53 tumour suppressor protein (12). The authors present data showing that MK5 phosphorylates S37 of p53, which lies within the transactivation domain *in vitro*. Interestingly, this phosphorylation site is not within a consensus phosphorylation site for MAPKAP kinases. Instead, S37 is followed by a proline making this site a more likely candidate for direct phosphorylation by a MAP kinase. This may suggest a more direct link between p38 and p53 without invoking any need for MK5. Overall these results are potentially very interesting and postulate a functional link between the p38 MAPK pathway and activation of MK5. It will be critical to follow up these initial studies in order to verify the involvement of p38 in this potentially important mechanism of tumour suppression.

A role for MK5 in the regulation of cytoskeletal organization has recently been reported (21, 25). Over-expression of MK5 in HeLa cells leads to an increase in both F-actin production and cell migration (25). In this study MK5 was also shown to interact with 14-3-3ε and co-expression of 14-3-3ε resulted in inhibition of MK5 kinase activity. Interestingly, co-expression of 14-3-3ε and MK5 also decreased the ability of MK5 to induce F-actin and cause increased cell migration. Conversely, siRNA-mediated knockdown of 14-3-3ε increased the kinase activity of over-expressed MK5 and also its ability to induce both F-actin and cell migration (25). In another study, treatment of PC12 cells with forskolin caused a transient increase in F-actin levels and this was blocked in PC12 cells transfected with an siRNA against MK5 (21). How MK5 mediates cytoskeletal rearrangement is still unclear, and further studies are needed to address this. Since MK2, MK3 and MK5 have overlapping substrate specificity and MK2 is known to phosphorylate several proteins involved in cytoskeletal organization (reviewed in (7)) it will be important to perform the studies described above in transgenic models lacking either MK2, MK3 or MK5.

Finally, a possible link between MK5 and neurological/cognitive function has been proposed based on behavioural analyses of a transgenic mouse that expresses a constitutively active mutant of MK5. This study revealed complex sex-specific changes in both anxiety-related traits and locomotor activity in MK5 expressing mice relative to WT controls (26). However, these results must be interpreted with caution as MK5 expression was under the control of the CMV promoter and the differences observed may reflect either inappropriate or over-expression of MK5 activity in tissues where endogenous MK5 is not normally present. It will be interesting to subject the MK5 knockout animals to similar analysis to see if loss of function has any effect on these endpoints.

8. CONCLUSIONS

The physiological function(s) of MK5 are only now beginning to receive serious attention following the generation of mice in which the MK5 gene has been deleted. Thus far it is clear that MK5, unlike MK2, does not play a critical role in mediating inflammatory and stress responses (11, 12). However, the embryonic lethality seen in inbred strains of mice lacking MK5 indicates that this kinase has important and essential functions (3). Much more work is required using these animals and cells derived from them in order to further dissect the links between MK5 and the signalling pathways which lie both upstream and downstream of this conserved MAPKAP kinase. At the present time, MK5 activity and localisation has been linked with both the p38 MAP kinase pathway and to the activities of the atypical MAP kinases ERK3 and ERK4 (1, 3-6). The relative roles of p38 and ERK3/4 in the regulation of MK5 activity require resolution. This could be addressed by

using mice lacking p38 MAP kinase isoforms and also mice in which ERK3 and ERK4 are deleted, when these become available. The role of MK5 in mediating a tumour suppressor function and how this might be integrated into our knowledge of both the p38 MAP kinase and its downstream effectors such as p53 must also be a key area for future study. Finally, as more is learnt about the regulation and physiological roles of the atypical MAP kinases ERK3 and 4, the importance of these hitherto little studied members of the MAP kinase family in mediating signalling via MK5 should become clear.

9. ACKNOWLEDGEMENTS

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Send correspondence to: Ole Morten Seternes, Department of Pharmacology, Institute of Medical Biology, University of Tromso, N-9037 Tromso, Norway, Tel: 477764-6506, Fax: 477764-5310, E-mail: olems@fagmed.uit.no

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