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Specificity of signaling from MAPKs to MAPKAPKs: Kinases' *Tango Nuevo*

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1. ABSTRACT

Within the signalling network of mammalian cells, MAPK-activated protein kinases (MAPKAPKs) have been identified downstream to various MAPKs, such as the classical MAPKs, ERK1/2, the stress-activated p38 MAPKs and the atypical MAPKs ERK3/4/5. Here, the current understanding of the specificity of MAPK to MAPKAPK signalling, the underlying mechanisms of protein-protein-interaction and the effects on subcellular localisation are reviewed. It is demonstrated that specificity in this signalling section is maintained by protein domain interactions and by regulated subcellular localisation. These mechanisms enable specific MAPK pathways to act independently via specific MAPKAPKs but also allow different MAPK pathways to cooperate in downstream signalling in a coordinated fashion.

2. SPECIFICITY IN ACTIVATION OF DOWNSTREAM KINASES BY DIFFERENT MAPKS

The existence of distinct MAPK cascades raises the question whether these linear cascades continue as separate signalling pathways downstream from their central elements, the MAPKs, or whether there is a broad overlap in substrates and functions of the different MAPKs. Here, this question will be analysed for the protein kinases downstream to MAPKs, generally designated MAPK-activated protein kinases (MAPKAPKs) (reviewed in (1) and in this special issue of Frontiers in Bioscience). MAPKAPKs consist of different subfamilies: the p90 ribosomal S6 kinases, RSKs, the mitogen- and stress-activated protein kinases, MSKs, the MAPK-interacting kinases, MNKs, and the MAPKAPKs MK2, MK3/3pK and

MK5/PRAK (reviewed in (2)). RSKs and MSKs carry two catalytic kinase domains while MNKs and MKs display a single kinase domain homologous to the C-terminal kinase domain of RSKs and MSKs.

It is known that *in vitro* and in cells that ectopically overexpress protein kinases, MAPKAKs can be activated by various MAPKs that share the minimal substrate consensus motif of proline-directed kinases. Consequently, MK2 is activated and phosphorylated *in vitro* at identical sites by ERK1 (3, 4), ERK2 (3, 5), and p38α (4), and MK5 is activated *in vitro* by ERK2 and p38α but, unexpectedly, not by JNK (6). Moreover, ectopic overexpression of MK3/3pK in 293 cells alone or together with overexpressed MAPKs and MAPKKs leads to their stimulation as a result of activation of each, the ERK-, the JNK- and the p38-cascade (7). However, the *in vivo* situation in specific activation of MAPKAPKs should be better analysed using MAPK inhibitors - when following the guidelines of their use carefully, such as analysing the concentration-dependence of inhibition and using different inhibitors with the same main specificity but different cross-inhibition of other kinases (8, 9) - as well as genetic models where MAPKs are deleted (reviewed in (10)).

In vivo, RSK activation is completely blocked by the MEK1/2 inhibitors U0126 and PD98059 (11) indicating an exclusive activation of RSKs by ERK1/2 in most cells. However, due to the sequential activation mechanism of RSK (see review of Carriere *et al.* in this special issue (72)), where ERK phosphorylation in the C-terminal kinase domain leads to autophosphorylation, generation of a PDK1-docking site and phosphorylation of the activation loop in the N-terminal kinase domain by PDK1, RSK1-3 activation is also blocked in PDK1 knockout cells (12). Interestingly, ERK5 also specifically binds to and phosphorylates RSK indicating that it might be also relevant for RSK activation *in vivo* (13). Unexpectedly, RSK-dependent macropinocytosis in dendritic cells (DC) could not be inhibited by the MEK inhibitors, but by RSK inhibitors, such as BI-D1870 or SL0101, or by MEK and p38 inhibitors together (14). This finding indicates that in DC there is also a p38-dependent mechanism of RSK activation. Indeed, the p38 activated MAPKAPKs MK2 and MK3 are able to directly phosphorylate RSK and generate the PDK1 docking site in DC. In this signalling scenario, RSK is downstream to the MAPKAPKs and should be regarded a "MAPKAPKAPK" or a kinase activated by MKs, "KMK" (14, 15). Very recently, it was demonstrated that a constitutively activated, cancerogenic FGF-receptor 3 directly phosphorylates RSK2 at Y529, which facilitates activation of RKS2 by ERKs (16). It will be interesting to see whether tyrosine phosphorylation plays a role also in activation of other MAPKAPKs.

In contrast to RSK, the sequential scenario of MSK activation is completely fulfilled by MAPKs and autophosphorylation without the help of PDK1 (see review of Arthur in this special issue (75)). Since *in vivo* activation of MSKs by complex stimuli, such as treatment with TNF, NGF or FGF, could only be blocked by parallel treatment with PD98059 and the p38α/β inhibitor SB203580, it became rapidly clear that these kinases can be activated by both groups of MAPKs (17).

For the MNKs, consisting of MNK1 and MNK2, there is no common mechanism of activation (see review of Buxade *et al.* in this special issue (76)). MNK1 activation could be completely inhibited only by the MEK and p38 inhibitors together, indicating that both classical MAPKs ERK1/2 and p38α/β can activate this enzyme (18). In contrast, MNK2 shows higher basal activity, binds relatively stable to phospho-ERKs and, due to this binding, MNK2 is relatively insensitive against MEK-inhibitors (19).

Activation of MK2 and MK3/3pK can be completely blocked by the inhibitor SB203580 indicating that these kinases are exclusively activated by p38α/β *in vivo* (20). In MEFs, p38α is the major activator of MK2, since genetic deletion of p38α in these cells leads to an almost complete inhibition of anisomycin- and arsenite-induced MK2 activation which cannot further be suppressed by SB203580 treatment (21). Consistently, in p38β-deficient MEFs only a very slight reduction of anisomycin-induced MK2 activity was observed (22).

For MK5/PRAK activation, the *in vivo* situation is controversial (see also review of Perander *et al.* in this special issue (73)). First of all, although primarily described as p38-regulated and -activated kinase PRAK (23), in MEFs MK5 seems not to be activated by p38-specific stress-stimuli such as arsenite or sorbitol, and does not display the same stable interaction with p38α as known for MK2 (24). So far, no stimulus for transient MK5 activation could be identified and the only conditions, where increased endogenous MK5 activity in cells is detected, is after Ha-RasV12-transformation induced cellular senescence (25). Since no transient induction of MK5 activity is known, the use of kinase inhibitors to identify its activators is not possible. When used in transformation and senescence assays, secondary effects of the kinase inhibitors will impede the interpretation of the results.

As a result of Y2H screens with MK5 as bait, the atypical MAPKs ERK3 and ERK4 were identified as molecules which stably interact with and activate MK5 (26, 27). Since regulation of activity of ERK3/4, which lack the classical MAPK activation loop motif TXY, is also enigmatic so far (reviewed in (28)), this pathway of MK5 activation is far from being understood.

3. PROTEIN DOCKING INTERACTION

It is assumed that the specificity in activation of MAPKAPKs by MAPKs described above (cf. Figure 1) is due to specific protein-protein interactions involving defined docking sites and domains within these enzymes. MAPKs display the common docking CD domain (29) prolonged by a specific two amino acid patch which is neutral in classical ERKs (TT-motif) and acidic in p38 (ED-motif) forming a docking grove (Figure 2) for their activator kinases, inactivating phosphatases and substrates,

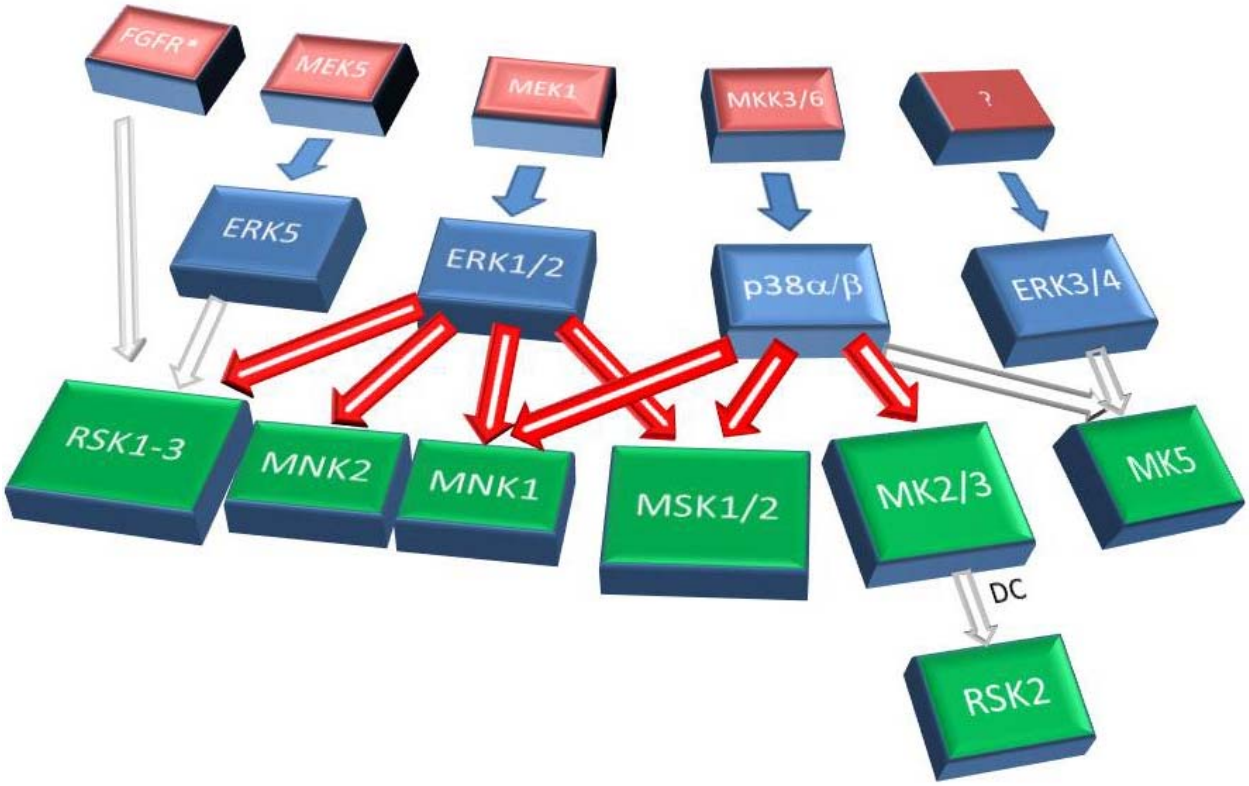

Figure 1. Specificity of MAPK signalling to MAPKAPKs.

such as MAPKAPKs (30). The importance of these docking interactions was nicely demonstrated by ED/TT motif swapping rendering activator specificity for MK3 from p38 to ERK (30). In addition, insertion of 8 amino acids in the p38 splice variant p38β2 not far from the ED-motif leads to complete loss of phosphorylation of docking-dependent substrates, such as MK2 and MK3, but does not influence activity of p38 against myelin basic protein (31). Interestingly, a substrate selective p38 inhibitor, which inhibits phosphorylation of MK2 but not of ATF2, allosterically interferes with the docking groove residues ED in p38 (32).

Complementary to the docking groove, binding partners of MAPKs carry a D motif or D site (29, 33) which fits into the docking groove and enables specific interaction with MAPKs (Figure 3). How can the D motif determine whether a MAPKAPK is activated via ERK1/2 or p38α/β? Obviously, an increase in number of basic core residues of the D motif facilitates binding to p38 vs. ERK1/2 (cf. Figure 3). Furthermore, in the D motif K/R-K/R-X_{2-6}-Φ-X-Φ, where Φ represents a hydrophobic residue, such as L, I, or V, the hydrophobic patch could also contribute to specificity. The recent elucidation of the three-dimensional structure of the p38α/MK2 complex (34, 35) underlines the intimate and specific binding between both molecules. Furthermore, this co-structure provides new information on how the D motif of MK2 interacts with the CD domain and the ED motif of p38α (Figure 4). Electrostatic interactions between K385, R386 and K389 of the D motif with D313 and D316 of the CD domain are obvious. In addition, K373 of the D motif can be identified as the major basic residue which interacts with the acidic ED (E160, D161) motif of p38α. This lysine residue (bold in Figure 3) is only present in MK2 and MK3 and could contribute to their exclusive activation by p38.

Beside the CD interaction, further regions in p38, such as the MK2-binding region from N114 to V127 (36), contribute to substrate specificity. Apart from this, other MAPK-binding motifs were also identified. The DEF (docking site for ERK: F/Y X F/Y P) motif is found in ERK substrates, such as the transcription factors c-fos, egr-1 and Elk1, and is responsible for binding to the ERK2 DEF docking domain located opposite to the CD-domain (37, 38). So far, no DEF motif has been identified in MAPKAPKs. Hence, interfering with CD- or DEF-interaction in a separate manner could specifically inhibit MAPK signalling to MAPKAPKs and transcription factors, respectively (38, 39). Interestingly, some ERK substrates, such as cAMP-specific phosphodiesterases, carry both a FGF-type DEF motif and a kinase interaction motif (KIM), which is similar to the D motif (40, 41) (cf. Figure 3).

A completely different interaction is responsible for docking between MK5 and ERK3/4. For this interaction the D motif of MK5 is not necessary, instead a motif within the C-terminus between amino acids 423-472 is needed (26, 27). Deletion mutants of ERK3 and ERK4 revealed that the region between amino acid 330 and 340 is essential for MK5 binding (26, 27, 42, 43). Interestingly, this region displays a degenerated CD domain where the first D is

ERK1	176	**T**	**T**	177	-	330	L	E	Q	Y	Y	**D**	P	T	**D**	E	P	V	A	E	343
ERK2	159	**T**	**T**	160	-	313	L	E	Q	Y	Y	**D**	P	S	**D**	E	P	I	A	E	326
ERK3	163	**E**	**D**	164	-	328	S	H	P	F	H	-	I	**E**	**D**	E	V	D	D	I	341
ERK4	159	**E**	**D**	160	-	325	Q	H	P	F	R	-	I	**E**	**D**	E	I	D	D	I	338
ERK5	191	**E**	N	192	-	347	L	A	K	Y	H	**D**	P	**D**	**D**	E	P	D	C	P	360
P38α	160	**E**	**D**	161	-	308	F	A	Q	Y	H	**D**	P	**D**	**D**	E	P	V	A	D	321
P38β	160	**E**	**D**	161	-	308	F	S	Q	Y	H	**D**	P	**E**	**D**	E	P	E	A	E	321
P38β2	168	**E**	**D**	169	-	316	F	S	Q	Y	H	**D**	P	**E**	**D**	E	P	E	A	E	329
P38γ	163	**E**	**D**	164	-	311	F	E	S	L	H	**D**	T	**E**	**D**	E	P	Q	V	Q	324
P38δ	160	**E**	**D**	161	-	308	F	E	P	F	R	**D**	T	**E**	**E**	E	T	E	A	Q	321

Figure 2. Primary structure of the docking groove of human MAPKs formed by TT/ED motif and CD domain.

MAPKAPK	D motif	Activated by
RSK1	716 SSKPTPQLKPIESSI**L**AQ**RR**V-RKLP**S**TTL 744	ERK1/2
RSK2	711 NRNQSPVLEPVGRST**L**AQ**RR**GIKKIT**S**TAL 740	ERK1/2
RSK3	712 RTPQAP**R**LEPVLSSN**L**AQ**RR**GMKRLT**S**TRL 741	ERK1/2
RSK4	716 HKTFQPVLEPVAASS**L**AQ**RR**SMKKRT**S**TGL 745	ERK1/2
MNK2	429 ATSRCLQLSPPSQSK**L**AQ**RR**QRASLSSAPV 458	ERK1/2
MNK1	387 DGLCSM**K**LSPPCKSR**L**A**RRR**ALAQAGRGED 416	ERK1/2, p38
MSK1	725 YKREGFCLQNVDKAP**L**A**KRRK**MKKT**S**T**S**TE 754	ERK1/2, p38
MSK2	712 GKREGFFLKSVENAP**L**A**KRRK**QKLR**S**ATAS 741	ERK1/2, p38
MK2	368 EQIKI**K**KIEDASNPL**L**L**KRRKK**ARALEAAA 397	p38
MK3	347 DQVKI**K**DLKTSNNRL**L**N**KRRKK**QAGSSSAS 376	p38
MK5	344 DLKVSL**K**PLHSVNNPIL**RKRK**LLGTKPKDS 373 434 DTLQSFSWNGRGFTDKVD**R**L**K**LAEIVKQVI 463	p38 ? ERK3/4
D motif	**RR**--ΦXΦ **KK**---ΦXΦ ----ΦXΦ -----ΦXΦ ------ΦXΦ	
KIM motif	**L**XX**RR**XXXXX**L** **V** **KK**	

Figure 3. D motifs and other potential docking motifs in MAPKAPKs and the binding MAPKs. Consensus sequences, basic residues in an ED-site complementary position and phosphorylation sites are in bold. Φ represents I, L or V.

missing (cf. Figure 2). Possibly, this motif binds to the incomplete D motif of MK5 located in the C-terminus between amino acids 434-463 (cf. Figure 3).

An intriguing possibility is the signal-dependent regulation of MAPK docking to its substrates. For example, ERK-mediated phosphorylation at S741 near the D motif of RSK (cf. Figure 3) results in impaired docking between RSK and ERK (44). Whether the autophosphorylation sites in the homologous region of MSK1 (S750, S752) and MSK2 (S737) (in bold in Figure 3) also regulate MAPK docking is not clear so far. Regardless, phosphorylations at the MAPKs themselves could both positively and negatively regulate docking: While phosphorylation of p38α by GRK2 in the docking groove inhibits its signalling to D motif substrates (45), phosphorylation of ERK4 at S186 allosterically stabilises its interaction with MK5 (46). Whether the specific TCR-dependent activation of p38 by phosphorylation of Y323 in the vicinity of the CD domain (47) alters docking specificity of p38 in T cells is still open.

4. DOCKING AND SUBCELLULAR LOCALISATION

While MSKs are constitutively nuclear protein kinases, RSKs, MNKs and MKs show various subcellular translocations which are probably essential for their proper function. After activation, cytoplasmic RSK1-3 translocate at least partially to the nucleus (48) or to the periplasmic membrane (49), while RSK4 stays predominantly in the cytoplasm (50). The molecular mechanisms for these translocations of RSK are not completely understood, and, to a certain degree, this situation resembles the lack of comprehensive understanding of ERK1/2 translocation (51). However, there is no doubt that regulated protein-

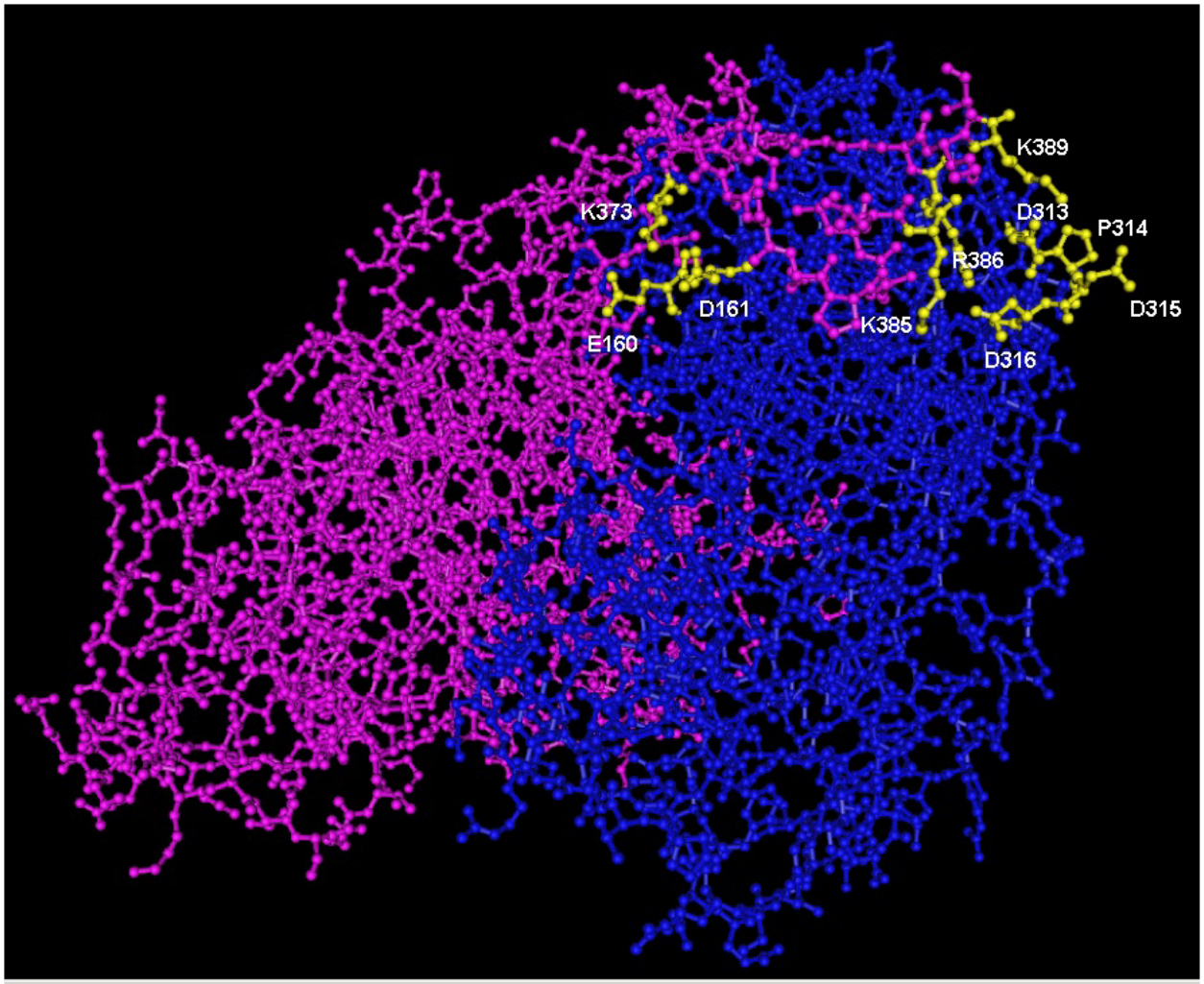

Figure 4. Molecular interaction of CD domain and ED motif of p38 with the D motif of MK2 as seen in the 3D structure published in (35). MK2 pink, p38α blue. Interacting residues are in yellow and labelled.

protein interaction is crucial for RSK translocations, such as docking to ERKs or PEA-15 (52).

For human MNKs, due to differential spliced isoforms, the situation is rather complex (53, 54). However, the ERK-binding long isoforms of MNK1/2 seem to constantly shuttle between cytoplasm and nucleus and, due to an efficient nuclear export motif (NES) in MNK1 and to C-terminal regulated binding to eIF4G for MNK2, are mainly localised in the cytoplasm of stimulated and non-stimulated cells.

Interestingly, MKs display MAPK-dependent translocations (reviewed in (2)). MKs carry a functional bi- (MK2) or mono-partite (MK3, MK5) nuclear localisation signal (NLS) within the D motif (cf. Figure 3) which directs them to the nucleus of resting cells. As a result of phosphorylation by p38α/β, GFP-MK2 and GFP-MK3 are exported from the nucleus to the cytoplasm (30, 55, 56). This is explained by a phosphorylation-dependent bi-functional switch within this molecule which simultaneously leads to a release of the autoinhibitory C-terminal domain from the catalytic core and unmasking of the C-terminal NES (57, 58). It has been described that p38 is co-exported in complex with MK2 from the nucleus (59), but the recent elucidation of the three-dimensional structure of the non-phosphorylated p38α/MK2 complex (34, 35), where the NES is completely masked by p38-binding, requires at least some dramatic phosphorylation-dependent opening of the complex. An alternative model would require dissociation of the complex in the nucleus and separate export of MK2 and p38 (see review by Ronkina *et al.* in this special issue (74)).

The overlap of D motif and NLS in MKs potentially couples MAPK-binding with inhibition of nuclear import and changes in subcellular localisation. Indeed, GFP-tagged MK3 is mainly detected in the cytoplasm when co-expressed with p38α or its kinase dead mutant, but not when co-expressed with a CD domain (DPDD->NPNN)/ED->TT mutant of p38α (30). This indicates that docking interaction regulates subcellular

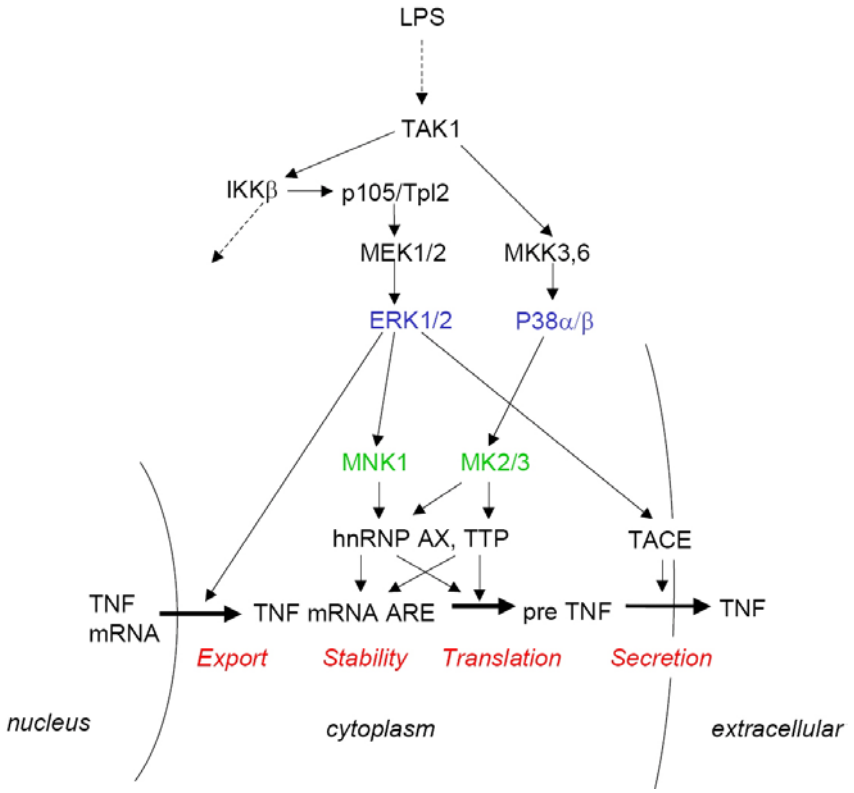

Figure 5. Cooperation between MAPKs (blue) and MAPKAPKs (green) in stimulation of TNF biosynthesis.

distribution of MK3 by masking its NLS. A similar D motif- and CD domain-dependent mechanism of cytoplasmic accumulation of overexpressed GFP-MK5, which shows nuclear localisation, by co-expression of p38β and, to a lower extent, also by p38α, has been reported (60). In contrast, cytoplasmic anchoring and activation of GFP-MK5 proceeds via co-expression of ERK3 or ERK4 in a D motif-independent manner (26, 27, 42, 43). Instead, the C-terminal part of MK5 containing the degenerated D motif (amino acids 434-463, cf. Figure 3) is necessary.

Here, it should be mentioned that many localisation studies used overexpressed and tagged kinases, which may vary in their subcellular localisation compared to the endogenous enzymes, as already demonstrated for MK5/PRAK (60). Hence, no final conclusions for localisation of the endogenous kinases in dependence on MAPK activity can be drawn as long as antibodies suited for subcellular immunocytochemistry of endogenous MK2 and MK3 are still lacking. However, the overexpression results strongly indicate that MAPK docking and MAPKAPK activation and subcellular localisation could be coupled within a complex manner of regulation.

5. PERSPECTIVE: SPECIFIC PATHWAYS WITH FUNCTIONAL CONVERGENCE IN INFLAMMATION

Although the activation of different MAPKAPKs by MAPKs is highly specific (see above), under certain conditions various MAPKs and MAPKAPKs have to cooperate to enable a complex cellular response. One of the best examples in this direction is the inflammatory response of macrophages to Toll-like receptor stimulation by, e.g. bacterial lipopolysaccharide (LPS) resulting in production and secretion of cytokines, such as TNF (Figure 5). Since it needs both MEK inhibitors and p38 inhibitors to completely block production of cytokines such as IL-1 (61) and MIP-2 (62), this response comprises activation of both, ERK and p38 pathway. As a result of activation of the central upstream kinase TAK1 and via different pathways (cf. Figure 5), both ERK1/2 and p38α/β are activated and, in turn, phosphorylate their MAPKAPKs, such as MNK1 and MK2/3, respectively. ERK1/2, MNK1 and MK2/3 then cooperate in regulation of cytokine expression at different posttranscriptional levels: ERK1/2, when activated by LPS via the Tpl2 pathway (63), promotes the transport of TNF mRNA from the nucleus to the cytoplasm (64). Many cytokine mRNA carry AU-rich elements (AREs) in their 3' non translated region which can bind specific proteins and regulate cytoplasmic stability and translation of these transcripts (65) (see review of Clark *et al.* in this special issue (77)). By phosphorylation of some of these TNF ARE-binding proteins, such as tristetraproline (66) or hnRNP A0 (62), MK2, and probably also MK3 (67), can stabilise the TNF mRNA and stimulate its translation (68, 69). Furthermore, MNK1 contributes to TNF-ARE regulation via phosphorylation of hnRNP A1 (70). After TNF mRNA is used for translation of pre-TNF protein, further processing and secretion is necessary for cytokine

signalling. Here, it was recently shown that TACE, the protease necessary to cleave pre-TNF to be secreted, is phosphorylated by ERK1/2 at T735 (71). This might stimulate TNF shedding by regulating the pre-TNF-TACE complex. Taken together, regulation of biosynthesis of cytokines, such as TNF, is a nice paradigm for concerted action of different MAPKs and MAPKAPKs, a cooperation which could be of importance for other biological processes as well.

It takes two to tango - MAPK and MAPKAPK. For the newer style *Tango Nuevo*, the "embrace is often quite open and very elastic, permitting the leader to lead a large variety of very complex figures" (Wikipedia) or, in more scientific language, transient or stable interacting pairs of specific MAPKs and MAPKAPKs influence the enzymatic activity and/or subcellular localization of both partners, thus enabling the cell to respond to various stimuli with complex behavior.

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