Substrate Specificity and Activity Regulation of Protein Kinase MELK*

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Maternal embryonic leucine zipper kinase (MELK) is a protein Ser/Thr kinase that has been implicated in stem cell renewal, cell cycle progression, and pre-mRNA splicing, but its substrates and regulation are not yet known. We show here that MELK has a rather broad substrate specificity and does not appear to require a specific sequence surrounding its (auto)phosphorylation sites. We have mapped no less than 16 autophosphorylation sites including serines, threonines, and a tyrosine residue and show that the phosphorylation of Thr167 and Ser171 is required for the activation of MELK. The expression of MELK activity also requires reducing agents such as dithiothreitol or reduced glutathione. Furthermore, we show that MELK is a Ca^{2+}-binding protein and is inhibited by physiological Ca^{2+} concentrations. The smallest MELK fragment that was still catalytically active comprises the N-terminal catalytic domain and the flanking ubiquitin-associated domain. A C-terminal fragment of MELK functions as an autoinhibitory domain. Our data show that the activity of MELK is regulated in a complex manner and offer new perspectives for the further elucidation of its biological function.

The AMP-activated protein kinase (AMPK) is the best characterized member of the subfamily of AMPK-related protein Ser/Thr kinases (1, 2). It is allosterically activated by AMP and controls processes that restore the energy charge in the cell. The other members of the subfamily of AMPK-related kinases include the protein kinases ARK5, BRSK, MARK, MELK, QIK, NUAK, and SNARK, which fulfill functions in processes as diverse as cell cycle progression, cell survival, cell differentiation, and microtubule stability (3–6). With the exception of maternal embryonic leucine zipper kinase (MELK), which is activated by autoprophosphorylation, the AMPK-related kinases are activated through phosphorylation of their T-loop by protein kinase LKB1 (5, 7) and the calcium- and calmodulin-dependent protein kinase II (8).

The catalytic domain of the AMPK-related protein kinases is located in the N terminus of their catalytic subunit. Some of the AMPK-related protein kinases, including MELK, have an ubiquitin-associated (UBA) domain adjacent to the catalytic domain and a C-terminal kinase-associated 1 (KA1) domain. The function of the latter domains are poorly understood. UBA domains are known to bind (poly)ubiquitin and have been suggested to thereby prevent additional ubiquitination and proteosomal degradation of the target protein (9–12). In some proteins, UBA domains appear to function as dimerization domains (13). Between the UBA and the KA1 domains, MELK contains a TP dipeptide-rich domain that is phosphorylated in mitotically arrested cells and mediates binding to the transcription and splicing factor NIP1 (14).

Preliminary evidence implicates MELK in various cellular processes. MELK binds tightly to the zinc finger-like protein ZPR9 and causes its nuclear accumulation (15, 16). In the nucleus, ZPR9 itself interacts with the transcription factor B-Myb, a regulator of cell proliferation and differentiation, and enhances its transcriptional activity. Another interactor of MELK is the transcription and splicing factor NIP1, but the binding of NIP1 requires the phosphorylation of MELK on a specific threonine in its TP dipeptide-rich domain (14). Because wild-type MELK, but not a NIP1-binding mutant, is a potent inhibitor of pre-mRNA splicing in nuclear extracts and because the MELK-NIP1 interaction is increased during mitosis, it has been proposed that MELK contributes to the ending of pre-mRNA splicing just before mitosis (14).

A third protein ligand of MELK is Cdc25B, a protein-tyrosine phosphatase that triggers mitosis by the activation of protein kinase Cdk1. Davezac et al. (17) reported that the ectopic expression of MELK induces an accumulation of cells in G2, and that this effect was counteracted by the overexpression of Cdc25B. Intriguingly, MELK is expressed at high levels in both embryonic (18, 19) and neural stem cells (20, 21), indicating that it may also play a role in stem cell functions of multipotency and self-renewal. Finally, MELK possibly contributes to oncogenesis because its expression is increased in tumor-derived progenitor cells (22) and in cancers of nondifferentiated cells (23).

The MELK ligands ZPR9 (15), NIP1, and Cdc25B (17) are also MELK substrates, but the significance of their phosphorylation is not clear. A major limitation in studying the role of MELK as a protein kinase is that it is not known what controls its activity and that MELK expressed in mammalian cells seems to be inactive (5). This has prompted us to examine what determines the activity and substrate specificity of MELK, expressed in either bacteria or mammalian cells. We show here that MELK has a rather broad substrate specificity and that its activity is complexly regulated by autoprophosphorylation, autoinhibition, Ca^{2+} ions, and reducing agents.

EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal anti-FLAG antibodies were obtained from Stratagene. Anti-phosphothreonine antibodies were obtained from Zymogen, and anti-phosphotyrosine antibodies were obtained from Santa

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5 The abbreviations used are: AMPK, AMP-activated protein kinase; KA1, kinase-associated 1 domain; MBP, myelin basic protein; MELK, maternal embryonic leucine zipper kinase; NIP1, nuclear inhibitor of protein phosphatase-1; UBA, ubiquitin-associated domain; DTT, dithiothreitol.

6 M. Beullens, V. Vulsteke, S. Vancauwenbergh, and M. Bollen, unpublished data.
Cruz Biotechnology. Reduced and oxidized glutathione and PHOS-Select™ gel were purchased from Sigma. 

Preparation of Recombinant MELK (Mutants)—Wild-type MELK and the indicated MELK mutants and fragments were cloned in the pET16b vector, in-frame with the polyhistidine tag. The His-tagged proteins were purified on Ni²⁺-Sepharose™ Fast Flow (Amersham Biosciences). The constructs encoding FLAG-tagged MELK (fragments) have been described previously (14). Point mutations were made according to the QuikChange site-directed mutagenesis protocol of Stratagene, using the appropriate primers and templates. The sequences of the DNA constructs were verified by DNA sequencing.

Cell Cultures and Immunoprecipitations—COS-1 and HEK293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. 48 h after transfection, the cells were washed twice with ice-cold phosphate-buffered saline and lysed in 50 mM Tris at pH 7.5, 0.3 M NaCl, 0.5% (v/v) Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine, and 5 μM leupeptin. After sonication, the cell lysates were cleared by centrifugation (10 min at 10,000 × g), and the supernatants were used for immunoprecipitations. The cleared cell lysates were incubated with anti-FLAG antibodies coupled to protein G-Sepharose (Amersham Biosciences) for 3 h at 10 °C. After one wash with Tris-buffered saline supplemented with 0.2 M LiCl and three washes with Tris-buffered saline plus 0.1% (v/v) Nonidet P-40, the beads were resuspended in 25 mM Tris at pH 7.5 and used for kinase assays and immunoblotting with anti-FLAG antibodies.

Protein Kinase Assays—The kinase activities of MELK (mutants) and fragments were determined with the indicated substrates for 1 h at 30 °C in a buffer containing 25 mM Tris at pH 7.5, 0.1 mM [γ-³²P]ATP, and 2 mM magnesium acetate. Reactions with peptide substrates were stopped by transfer of the assay mixture to P81 papers and washing with 75 mM orthophosphoric acid. Reactions with myelin basic protein (MBP) as substrate were run on SDS-PAGE and analyzed by autoradiography.

Peptide Chip—The Trial PepChip kinase array (Pepscan Systems B.V.) contains 2 × 192 peptides, divided in 2 × 4 subarrays of 6 × 4 spots. The incubation was done as described in the manual. Briefly, the PepChip kinase slide was incubated for 2 h at 30 °C in the following reaction mixture: 50 mM Hepes at pH 7.4, 20 mM MgCl₂, 0.02 mg/ml bovine serum albumin, 0.01% Brij-35, 20 mM DTT, 10 μM [γ-³²P]ATP, and 0.5 μM MELK. After the incubation, the chip was washed once with phosphate-buffered saline supplemented with 1% (v/v) Triton X-100, washed twice with 2 M NaCl plus 1% (v/v) Triton X-100 and then rinsed twice with Milli Q water before drying. The dry PepChip kinase array was analyzed on a phosphorimaging device (Storm 640; Molecular Dynamics).

Ca²⁺ Overlay and Ca²⁺ Buffer—The indicated amounts of MELK or NIPP1 were spotted on a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences), and the Ca²⁺ binding was carried out as described previously (24). The free Ca²⁺ concentration in bath solutions was calculated with the CaBuf program (Droogmans G.,...


KULeuven), taking into consideration the values of pH, temperature, ionic strength, association constants, and Mg²⁺ concentration.

Mapping of Autophosphorylation Sites of MELK—Phospho-amino acid analysis by thin-layer chromatography was done as described previously (25). MELK-(1–340) was incubated for 3 h at 30 °C in a buffer containing 25 mM Tris at pH 7.5, 0.1 mM [γ-³²P]ATP, and 2 mM magnesium acetate to allow complete autophosphorylation. The autophosphorylation sites of ³²P-labeled MELK-(1–340) were determined by phosphopeptide sequencing as well as by mass spectrometry. For this purpose, ³²P-labeled MELK-(1–340) was first subjected to a reduction for 45 min at 60 °C with 10 mM DTT and then to an alkylation for 45 min at room temperature in the dark with 35 mM iodoacetamide. The remaining iodoacetamide was neutralized by an incubation with 15 mM DTT for 45 min at room temperature in the dark. Subsequently, the proteins were precipitated with 10% trichloroacetic acid, and the aceton-washed pellet was digested overnight with 5 µg/µl trypsin in 200 mM ammonium bicarbonate plus 0.1% RapiGest (Waters). The resulting peptides were separated on a µRPC C2/C18 SC2.1/10 column (SMART System; Amersham Biosciences), equilibrated in 0.1% trifluoroacetic acid, and eluted with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The radioactive fractions were either analyzed by a 492 Procise (Applied Biosystems) amino acid sequencer, operated in the pulsed liquid mode, or by electrospray mass spectrometry on an Applied Biosystems API 3000 tandem mass spectrometer.

FIGURE 2. Mapping of active MELK fragments. A, domain structure of MELK showing the catalytic, UBA, TP dipeptide-rich, and KA1 domains. B, domain structure of MELK fragments and mutants. C, kinetic parameters for the MELK mutants illustrated in B, using the SAMS peptide or MBP as substrates. The $K_m$ is expressed in µM. The $K_{cat}$ is expressed in units/µmol of kinase (mutant). The results in C represent the means ± S.E. for 6–11 experiments. The asterisk refers to a significant difference as compared with the value for wild-type MELK ($p < 0.02$; Student's t test).
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spectrometer. The latter was equipped with a Protana nanospray source. Identification of the phosphopeptides and determination of the phosphorylation sites were performed using the neutral loss scan mode and the product ion scan mode, respectively.

The autophosphorylation sites of full-length MELK-(1–651) were also determined by mass spectrometry, using a different protocol. Wild-type MELK was digested in-gel with trypsin (5 µg/ml) in ammonium bicarbonate plus 0.1% N-octyl-glucoside as described previously (5). Digests were diluted to 0.1 ml with PHOS-Select™ wash/bind buffer (0.25 M acetic acid, 30% acetonitrile) and supplemented with 30 µl of a 10% (v/v) PHOS-Select™ gel, equilibrated in the wash/bind buffer. After an incubation for 20 min, the beads were captured in an Eppendorf gel loader tip to form a mini-column. The beads were washed three times with 30 µl wash/bind buffer and eluted with two times 25 µl of 0.4 M ammonium hydroxide. The eluate was dried under vacuum and resuspended in 5% formic acid for analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry on an Applied Biosystems 4700 Proteomics Analyser using α-cyano-4-hydroxy cinnamic acid (5 mg/ml + 10 mM ammonium phosphate in 50% acetonitrile, 0.1% trifluoroacetic acid) as matrix. Electrospay liquid chromatography-mass spectrometry was performed on a Dionex Ultimate capillary high pressure liquid chromatography system coupled to an Applied Biosystems 4000 Q TRAP mass spectrometer. The peptides were separated on a PepMap C18 column equilibrated in 0.1% formic acid/water and developed with a discontinuous acetonitrile gradient at 350 µl/min.

RESULTS

MELK Has a Broad Substrate Specificity—Consistent with a previous report by Lizcano et al. (5), bacterially expressed human MELK was spontaneously active and phosphorylated the AMARA peptide, a classical substrate of the subfamily of AMPK-related protein kinases (Fig. 1A). Another widely used substrate for these kinases, the SAMS peptide, was an even better in vitro substrate of MELK. Surprisingly, MELK also phosphorylated a broad range of structurally unrelated proteins, such as MBP, Histone H1, and the splicing factors CDC5L, NIPP1, and SAP155 (not shown). To obtain some insights into the substrate determinants of MELK, we screened a peptide chip for candidate substrates (Fig. 1B). The used chip contained two identical sets of 192 peptides that all com-2
strates were peptides derived from casein, eukaryotic initiation factor 2α, and lamin B1. These peptides did not show any structural similarity to the AMARA and SAMS peptides (Fig. 1B). Using smaller MELK fragments, we could delineate an inhibitory fragment to residues 326–530, which largely corresponds to the KP-rich domain (Fig. 2A). MELK-(602–651), essentially comprising the KP-rich domain, was not inhibitory. The latter finding was unexpected because the deletion of the KA1 domain increased the catalytic efficiency of MELK (Fig. 2C). Lineweaver–Burk plots showed that the inhibition of MELK-(1–340) by MELK-(326–601) was largely accounted for by a decreased Vmax (Fig. 3B), consistent with the increased Kcat for MELK-(1–340) as compared with the Kcat of wild-type MELK (Fig. 2C). The inhibition of MELK-(1–340) by MELK-(326–651), MELK-(326–601), and MELK-(326–530) was also detected when the SAMS peptide was used as substrate, but the maximal extent of inhibition was less pronounced with this substrate and amounted to only about 65% (not shown).

Autophosphorylation of MELK—Using immunoblotting with phospho-epitope-specific antibodies and phospho-amino acid analysis by thin-layer chromatography, we found that bacterially expressed MELK was phosphorylated on serine, threonine, and tyrosine residues and that the level of phosphorylation was further augmented by incubation of the purified enzyme with MgATP (not shown). The phosphorylation on Ser and Thr could be reversed by incubation with the catalytic subunit of protein phosphatase-1, whereas the phosphorylation on tyrosine was reversed by incubation with a protein-tyrosine phosphatase from Yersinia enterocolitica (not shown). The phosphorylation of MELK is
likely to be an autocatalytic process because the inactive variant MELK-D150A (14), which is mutated in the essential DFG triplet of kinase subdomain VII, was not phosphorylated at all.

We have initially used mass spectrometry to map the autophosphorylation sites of bacterially expressed wild-type MELK and MELK-(1–340). This resulted in the identification of 14 autophosphorylation sites, distributed all over the polypeptide chain, with the exclusion of the UBA and KA1 domains (TABLE ONE). Surprisingly, two independent mass spectrometric analyses did not identify tyrosine-phosphorylated peptides, which was in contrast with the data of immunoblot analysis. Because MELK contains a tyrosine in its catalytic loop (Tyr163) that is not conserved in the catalytic loop of the other members of the subfamily of AMPK-activated protein kinases (Fig. 4A) and because MELK is also the only member of this subfamily that is known to be autophosphorylated on tyrosine, we have examined whether it is perhaps Tyr163 that is autophosphorylated by MELK. In accordance with this view, we found that the autophosphorylation on tyrosine was largely lost in MELK-Y163F, despite the fact that this mutant was still catalytically active toward an exogenous substrate (Fig. 4B). We have also used N-terminal sequencing by Edman degradation to map autophosphorylation sites in the catalytic loop. A tryptic peptide that was derived from 32P-labeled MELK-(1–340), was phosphorylated on both Thr167 and Ser171 (not shown). The latter site was not identified by mass spectrometry, possibly because Ser171 was phosphorylated substoichiometrically (see "Discussion"). In conclusion, the total number of autophosphorylation sites that we mapped amounted to 16 (Fig. 4C).

MELK did not phosphorylate the SAMS peptide when the phospho- rylatable residue (Ser79) was replaced by a tyrosine (not shown), indicating that MELK is not a true dual specificity protein kinase and can only phosphorylate tyrosine(s) autocatalytically. Also, the autophosphorylation rate of MELK was independent of its dilution (not shown), suggesting that the autophosphorylation represents an intramolecular rather than an intermolecular process. In further agreement with this view, we found that wild-type MELK neither phosphorylated the recombinant (inactive) catalytic domain of MELK nor a synthetic peptide (residues 159–173) comprising the catalytic loop of MELK and including the autophosphorylation sites Tyr163, Thr167, and Ser171 (not shown).

We have subsequently explored the role of autophosphorylation of the catalytic domain of MELK by site-directed mutagenesis (Fig. 5). MELK-(1–340)-T167A was inactive, strongly suggesting that autophosphorylation on this site is essential for activity. However, MELK-(1–340)-T167D was active, indicating that this replacement mimicked the phosphorylation of Thr167. Interestingly, both MELK-(1–340)-S171A and MELK-(1–340)-S171D were inactive, suggesting that phosphoryl-

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### TABLE ONE

Mapping of autophosphorylation sites by mass spectrometry

| Phosphopeptide | Residues | Mass   | Phosphorylated residue |
|----------------|---------|--------|------------------------|
| IKpTEIREALK   | 54–62   | 1123.6 | Thr56                  |
| DpTEIRALK     | 54–65   | 1508.4 | Thr56                  |
| DpHQLpTCGSLAYAAPLEGQK | 162–183 | 2670.2 | Thr160                 |
| GNKDYHLPpTCGSLAYAAPLEGQK | 159–183 | 2969.5 | Thr160                 |
| RpSMK          | 251–255 | 713.6  | Ser131                 |
| KRlpSMK        | 250–255 | 841.6  | Ser153                 |
| LSqSFSCQASAPFTDK | 334–351 | 2043.9 | Ser176                 |
| LSqSFSCQApSAPFTDK | 334–351 | 2123.9 | Ser176, Ser177         |
| NNPSlEDVTASDK  | 352–365 | 1644.7 | Ser177                 |
| TpSQQTFNYWpTESNGVESK | 390–406 | 2150.9 | Ser177, Thr178        |
| YNPtESNGVESK   | 396–406 | 1378.6 | Thr178                 |
| pSLTPALK      | 407–404 | 1044.5 | Ser179                 |
| pSAVKNKEYFMFPRPK | 431–445 | 1894.9 | Ser179                 |
| LMPqGqVISpER   | 492–501 | 1118.6 | Thr179                 |
| CRPsGVDLDLNQAIMETPK | 503–520 | 2284.0 | Ser175                 |
| VFGpSLER       | 526–532 | 886.4  | Ser179                 |
| GLDKVipTVLR    | 533–543 | 1293.7 | Thr178                 |

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**FIGURE 4.** Mapping of the autophosphorylation sites of MELK. A, alignment of the T-loop sequence of MELK with the consensus T-loop sequence of the subfamily of AMPK-related protein kinases. $\delta$ represents a large hydrophobic residue; $s$, $n$, $g$, and $a$ represent preferences for Ser, Asn, Gly, and Ala, respectively; and $X$ is any amino acid. B, the left panel shows the autophosphorylation of MELK-(1–340) and MELK-(1–340)-Y163F on tyrosine, as detected by immunoblotting with anti-phosphotyrosine antibodies. The right panel shows the phosphorylation of MBP by MELK-(1–340) and MELK-(1–340)-Y163F, as visualized by autoradiography. C, schematic overview of the 16 mapped autophosphorylation sites of MELK wt, wild type.
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Mutation of Ser171 is essential for activity but is not mimicked by an acidic residue. Mutation of Thr56 or Ser253 into either an aspartic acid or an alanine did not measurably affect the activity of MELK-(1–340). Mutation of Tyr163 into either a phenylalanine or an aspartic acid residue also had no effect on the activity of MELK-(1–340).

The Expression of MELK Activity Requires Reducing Agents—The ability of MELK to autophosphorylate and to phosphorylate an exogenous substrate was completely dependent on the presence of reducing agents (Fig. 6A). A maximal stimulation of the protein kinase activity was obtained with 5–10 mM DTT. GSH was a less potent activator than was DTT, and GSSG did not activate MELK at all. The requirement for reducing agents suggested that MELK is inactivated by the covalent modification of one or several cysteines. The involved cysteine(s) are at least partially located in the catalytic and/or UBA domains because MELK-(1–340) was also fully DTT-dependent (Fig. 6B). Accordingly, the combined mutation of all nine cysteines yielded a MELK-(1–340) variant that no longer required reducing agents to be active (Fig. 6B). However, MELK remained DTT-dependent following the separate mutation of Tyr163 into either a phenylalanine or an aspartic acid residue also had no effect on the activity of MELK-(1–340).

FIGURE 5. Mutation of the autophosphorylation sites in the catalytic domain. Protein kinase activities of MELK-(1–340) and the indicated mutants with 100 μM of the SAMS peptide as substrate are shown. The activities are expressed as percentages of the activity of wild-type MELK-(1–340) and represent the means ± S.E. for four experiments.

FIGURE 6. Regulation of MELK by reducing agents. A, autoradiogram showing the autophosphorylation of MELK-(1–651) and the phosphorylation of MBP in the presence of the indicated concentrations of DTT, GSSG, and GSH. B, phosphorylation of MBP and autophosphorylation of MELK-(1–340) and MELK-(1–340)-C29V/C70V/C89A/C154A/C168A/C169A/C204A/C286A/C339A, denoted as the MELK-(1–340) cysteine mutant in the figure. The assays were performed in the absence or presence of 20 mM DTT, and the (auto)phosphorylation was detected by autoradiography.
MELK expressed in mammalian cells has similar properties. FLAG-MELK-(1–651), FLAG-MELK-(1–651)-T167D, and FLAG-MELK-(1–651)-T167A were expressed in HEK293T cells and immunoprecipitated from the lysates with anti-FLAG antibodies. The immunoprecipitates were assayed for protein kinase activities with 100 μM SAMS peptide as substrate. The activity of wild-type (wt) FLAG-MELK was also determined in the absence or presence of 20 μM DTT and in the presence of the indicated free Ca2+ concentrations.

DISCUSSION

Substrate Specificity of MELK—Most protein kinases have a rather restricted substrate specificity that is largely determined by the nature of the residues that surround their phosphorylation sites (26). However, MELK phosphorylated an unusually large fraction of the tested (poly)peptides, and the sequences flanking the phosphorylation sites did not conform to a consensus site (Fig. 1). Neither could a consensus sequence be delineated for the 16 mapped autophosphorylation sites (TABLE ONE). Our data therefore suggest that MELK has a rather broad substrate specificity in vitro. This does not necessarily imply, however, that MELK has also numerous substrates in vivo. For example, it cannot be excluded that MELK interacts with proteins that function as substrate specifiers, similarly to the well established regulation of protein phosphatase-1 (27).

Functions of the Noncatalytic Domains—The minimal MELK fragment that was catalytically active consists of the catalytic domain and the UBA domain (Fig. 2). Also, point mutations within the UBA domain of full-length MELK yielded a completely inactive enzyme. These findings were unexpected because UBA domains have thus far only been implicated in (poly)ubiquitin binding (9–12) and in the dimerization of proteins (13). Our data represent the first reported evidence that a UBA domain is required for the expression of catalytic activity by a protein kinase. Because UBA domains are also present in other members of the AMPK-related kinases, e.g. the MARKs (6), it will be important to examine whether they fulfill the same essential function in these kinases as well. The UBA domain is a small domain of about 40 residues that folds into a characteristic three-helix bundle (11). It is difficult to predict exactly how this domain contributes to the expression of kinase activity. The UBA domain does not appear to be involved in the dimerization of MELK because gel filtration studies indicated that MELK is a monomer.
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(not shown). It is possible that the UBA domain has a structural function and is important for the correct folding of the catalytic domain.

The C-terminal half of MELK, comprising the TP dipeptide-rich and KA1 domains, clearly functions as an autoinhibitory domain (Figs. 2 and 3). Using smaller fragments we could map the major inhibitory region to the TP dipeptide-rich domain. The KA1 domain alone was not inhibitory, but the TP dipeptide-rich domain was also a less potent inhibitor than the entire C-terminal half of the kinase, suggesting that the KA1 domain also contributes to autoinhibition in intact MELK. This conclusion is in accordance with a recent report on the yeast AMPK-related family members Kin1 and Kin2, which also have a C-terminal KA1 domain that was found to be autoinhibitory and to interact with the N terminus of the kinases (28).

Regulation by (Auto)Phosphorylation—MELK is a unique member of the AMPK-related protein kinases in that it does not require an upstream protein kinase for activation and is fully activated by autophosphorylation (Ref. 5 and this work). By various experimental approaches we have identified no less than 16 autophosphorylation sites, i.e. five in the catalytic domain and nine in the TP-rich domain and its flanking sequences. Surprisingly, two of these phosphorylation sites (Tyr163 and Ser171) were not identified by mass spectrometry, possibly because MELK was phosphorylated subsitiochemically on these sites. Other groups have also noted that the detection of all phosphorylation sites in a protein remains a most challenging analytical task, even when sophisticated techniques of mass spectrometry are adopted (29, 30). In any case, our findings nicely illustrate the importance of using multiple, independent approaches for the mapping of phosphorylation sites.

Site-directed mutagenesis indicated that Thr167 and Ser171, located between the DFG and APE motifs in the activation loop or T-loop, need to be autophosphorylated for MELK to be active as a protein kinase (Fig. 5). These sites are conserved in all other AMPK-related protein kinases (Fig. 4A), and the site corresponding to Thr167 has been shown to be phosphorylated by protein kinase LKB1 (5). It is not yet known whether the site corresponding to Ser171 is also phosphorylated in other AMPK-related protein kinases. Intriguingly, the phosphorylation of Ser171 could not be mimicked by an acidic residue.

MELK also autophosphorylates its T-loop on Tyr163 (Fig. 4B), a site that is not conserved in other AMPK-related protein kinases (Fig. 4A). In this respect, MELK is similar to the dual specificity tyrosine phosphorylation-regulated protein kinases (DYRKs), because they also autophosphorylate their activation loop on a tyrosine but phosphorylate their substrates on serine and threonine (31). It has recently been demonstrated that the tyrosine autophosphorylation by DYRKs is mediated by a translational intermediate and represents an essential step in the maturation of the kinase (31). Once DYRKs are fully translated and released from the ribosome, the transitional tyrosine kinase activity is lost, and they function as protein Ser/Thr kinases. This mechanism may therefore not apply to MELK, however, because autophosphorylation on Tyr163 is not essential for its activation (Fig. 5), and mature, bacterially expressed MELK also autophosphorylates on Tyr (not illustrated).

We have not yet explored the role of autophosphorylation of nine residues in the C-terminal, autoinhibitory domain (Fig. 4C). An enticing hypothesis is that these autophosphorylations decrease the inhibitory potency of this domain and thereby contribute to the activation of the kinase. It should be pointed out, however, that the autoinhibitory domain also contains numerous consensus phosphorylation sites for other protein kinases (14). For example, the TP-rich domain harbors 10 consensus phosphorylation sites for proline-directed protein kinases and the phosphorylation of one of these sites, Thr276, in mitotically arrested cells mediates the recruitment of the transcription and splicing factor NIPPI. It has not yet been investigated whether the binding of NIPPI affects the activity of MELK.

Regulation by Reducing Agents—In addition to its regulation by autoinhibition and autophosphorylation, the N-terminal half of MELK is controlled by oxidation of at least two cysteines (Fig. 6). Importantly, the dependence on reducing agents also applies to MELK that is expressed in mammalian cells and is probably the major reason for previous failures to detect kinase activities associated with MELK in mammalian cell lysates. There are many types of cysteine modification (32–34), and we currently do not know which one pertains to MELK.

Regulation by Ca2+—Finally, MELK is inhibited by free Ca2+ concentrations that are detected after the addition of optimal concentrations of a Ca2+ agonist (Fig. 7). Remarkably, supraphysiologically Ca2+ concentrations were less inhibitory, which is possibly accounted for by the existence of a second, lower affinity binding site for Ca2+ that opposes the effect of the binding of Ca2+ to the inhibitory, high affinity binding site.

MELK is the only member of the AMPK-related kinases that is known to be directly regulated by Ca2+. Overlay assays suggested that Ca2+ binds to the catalytic domain of MELK (Fig. 7B), but its primary structure does not contain an established Ca2+-binding motif. Therefore, additional work is needed to map the Ca2+-binding motif of MELK in more detail.

In conclusion, our initial failure to detect a MELK-associated kinase activity has led us to examine in some detail the determinants for the activity of MELK as a protein kinase. As a result of these studies we have shown here that MELK is complexly regulated by autoinhibition, (auto)phosphorylation, reducing agents, and free Ca2+. These data will be helpful in studying the physiological regulation and functions of MELK.

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