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Isolation and Characterization of *pmk*-(*1-3*), Three p38 Homologs in *C. elegans*

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Abstract

p38, a member of the mitogen-activated protein kinase (MAPK) superfamily, is activated in response to a variety of cellular stresses and ligands. Since the genome of the nematode *C. elegans* has been sequenced, we sought to identify and characterize the nematode homolog of mammalian p38. By sequence analysis and RT-PCR, we isolated cDNAs encoding three kinases, PMK-1, PMK-2, and PMK-3, which we call p38 map kinases due to their high sequence identity with p38. The three genes are contiguous on chromosome IV and comprise an operon. By use of a GFP reporter, we found that the promoter of the *pmk*s is active throughout the intestine. An active form of MAPK/ERK Kinase 6 (MEK6) phosphorylated and activated recombinant PMK-2 and PMK-3 *in vitro*. PMK-2 and PMK-3 phosphorylated Activating Transcription Factor-2 (ATF-2), indicating an activity similar to mammalian p38. When transfected into mammalian cells, these kinases, like p38, are stimulated by osmotic stresses.

Keywords

MAP kinase; MEK; stress response; MEKK

Introduction

The mitogen-activated protein kinases (MAPKs) comprise a family of serine/threonine kinases that function in a wide variety of biological processes. MAPKs are activated by tyrosine and threonine phosphorylation catalyzed by MAPK/ERK kinases (MEKs). The MEKs themselves are activated when they are phosphorylated by MEK kinases (MEKKs). These kinase cascades respond to an array of extracellular stimuli such as growth factors and environmental stressors.

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Extracellular-signal Regulated Kinases (ERKs), Stress-Activated Protein Kinases/c-jun N-Terminal Kinases (SAPK/JNKs), and p38 are the three best characterized subfamilies of MAP kinases and have representatives in all eukaryotes. Genetic analysis of *Drosophila* and *C. elegans* has revealed that MAPK pathways are necessary for normal development. The ERK pathway in *Drosophila*, which includes D-Raf, the MEK1/2 ortholog D-sor1, and the ERK ortholog Rolled, mediates cell signals important for the differentiation of photoreceptor cells (1) among others. In *C. elegans*, the ERK1/2 pathway, consisting of LIN-45, MEK-2, and the ERK ortholog MPK-1, plays an important role in vulval development (2). More recently, components of the JNK pathway, *jkk-1* and *jnk-1*, have been identified in *C. elegans* as part of a neuronal pathway controlling coordinated movement (3). Also, *mek-1*, a *C. elegans* homolog of a MEK in stress-sensitive pathways, plays a role in a nematode stress response (4).

The MAP kinase p38 is activated in response to various ligands and environmental conditions, such as hyperosmolarity, tumor necrosis factor (TNFα), and lipopolysaccharide (LPS) (5). Consistent with its functions in mammals, mammalian p38 complements a deletion of the yeast MAP kinase that mediates the response to glycerol (5). The kinase is inhibited by the pyridinyl imidazole SB203580 (6). Overexpression and *in vitro* experiments in mammals reveal that p38 can be activated by MEKs 3,4, and 6 (7). Here, we identify and characterize three p38 isoforms in *C. elegans* termed p38 Map Kinases 1-3 (PMK-1, PMK-2, and PMK-3). Interestingly, these three proteins appear to lie within an operon, allowing for the transcription of three highly related proteins from a single promoter. Their single promoter is active throughout the length of the intestine and the kinases are activated by osmotic stresses when expressed in mammalian cells. PMK-2 and PMK-3, which share the greatest identity to mammalian p38, are selectively phosphorylated and activated by only one of the three MEK family members that recognize mammalian p38s.

Materials and Methods

Cloning and Expression of p38 Homologs

Whole worm RNA was isolated using the Tri-reagent (Molecular Bioresources) and the RNA was subsequently used for RT-PCR with the cDNA cycle kit (Invitrogen) to isolate the cDNAs encoding PMK-1,2, and 3. Each of the cDNAs was amplified and inserted into the expression plasmids pRSET (Invitrogen) and either pCMV5-HA or pCMV5-$(HA)_3$. All cDNAs were sequenced to ascertain that no errors occurred in the amplification. For splice leader 2 (SL2) primed RT-PCR, the sequence of the SL2 primer used was GGTTTTAACCCAGTTACTCAAG (8).

Expression in HEK 293 cells was achieved with calcium phosphate transfection (9). Cells were harvested 48 hours post transfection in lysis buffer (50 mM Tris, pH 8.0, 1% Triton X-100, 100 mM NaCl, 5 mM EDTA, 1 mM Na-orthovanadate, 50 mM β-glycerophosphate) containing a cocktail of protease inhibitors (100 μM PMSF, 50 μg/ml each aprotinin, leupeptin, pepstatin A). Cells were stimulated by incubation in medium containing either 0.5 M sorbitol or 0.7 M NaCl for 10 minutes prior to harvest.

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Recombinant $(His)_6$-PMK-1, $(His)_6$-PMK-2, and $(His)_6$-PMK-3 were purified from the BL21 strain of *E. coli* after four hours of induction with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were lysed by sonication in 50 mM sodium phosphate, pH 8, supplemented with the protease inhibitors noted above. After centrifugation at 30,000 × g for 30 minutes, the supernatants were incubated with Ni^{2+}-NTA agarose beads. The resin was washed in buffer containing 0.3 M NaCl, 50 mM NaH_2PO_4, and 20 mM imidazole. Proteins were then eluted in pH 7.0 buffer containing 200 mM imidazole, 0.3 M NaCl, and 50 mM NaH_2PO_4.

Kinase Assays

For immune-complex kinase assays, immunoprecipitation was performed by adding anti-HA antibody and protein A-Sepharose beads to cell lysates for 2 hours. The sedimented beads were washed 3 times in a buffer containing 0.75 M NaCl, 20 mM Tris, pH 8.0 and once in a buffer containing 20 mM Tris, pH 8.0, 10 mM $MgCl_2$. Kinase assays contained 10 mM $MgCl_2$, 30 mM Tris, pH 8.0, 100 μM ATP ([γ-^{32}P]ATP, 2-7 cpm/fmol), and 1 mM dithiothreitol (DTT). Reactions were stopped by the addition of electrophoresis sample buffer and analyzed by SDS/PAGE and autoradiography.

For linked kinase assays, 200 ng of recombinant $(His)_6$-TAO1(1-416) was incubated with 50 ng of the indicated MEK proteins for 45 minutes at 30°C; 15% of this reaction was added to the second reaction containing the recombinant PMKs. 15% of this reaction was then transferred to a third reaction containing GST-ATF-2 at 10 μg/ml.

Recombinant DNA for GFP Expression

PCR from genomic DNA was employed to amplify a 3 kb fragment that included 1.5 kb upstream of the start codon of *pmk-1* and extended into the third exon of this gene. This PCR product was inserted into pPD95.81 at its SphI and BamHI sites. A second construct that extended into the last exon of *pmk-1* was also made and inserted into pPD95.75. Plasmids pPD95.81 and pPD95.75 were kindly provided by A. Fire (10). PCR was used to generate a fragment extending from 1.5 kb upstream of the start codon of *pmk-1* to the last exon of *pmk-2*. This piece was fused to GFP by overlap extension PCR for expression analysis of *pmk-2*. Wild type (Bristol N2) worms were injected with the constructs at 75 ng/μl along with an equivalent amount of the *rol-6(d)*-containing plasmid, pRAK3 (10,11). Worms were maintained by usual methods and grown on a lawn of the HB101 strain of *E. coli*.

The strains used were DA1750 *adEx1750* [*pmk-1*: :GFP *rol-6(d)*] and deletion strains DA1755 *pmk-1(ok169) IV* and VC36 *unc-5(gk29) pmk-2(gk21) IV/nT1(IV)*; *+/nT1(V)*] The deletion strains were obtained from the gene knockout consortium.

Results

The C. elegans Genome Encodes Three Protein Kinases Highly Related to p38

Genes encoding four mammalian p38 family members (α, β, γ, δ) have been identified (7). Database searches with their sequences reveals three previously uncharacterized open

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reading frames (ORF) of *C. elegans* that have significant similarity to mammalian p38. They are contiguous on chromosome IV. RT-PCR was employed to clone cDNAs that encode these three kinases, termed p38 map kinases 1-3 (PMK-1, PMK-2, PMK-3). (Accession numbers are PMK-1, T32642; PMK-2, T32643; PMK-3, T29750.) PMK-3 shares the highest identity with p38 family members while PMK-1 has the least. PMK-3 is approximately 60% identical and 75% similar to p38β while PMK-2 is 50% identical and 65% similar and PMK-1 is 45% identical and 60% similar (12) (Figure 1). The cDNA sequences obtained for *pmk-1* and *pmk-2* are shorter than the sequences predicted from the *C. elegans* genome database (ACeDB) (13). For both *pmk-1* and *pmk-2*, blocks of amino acids in the genefinder predictions not found in mammalian p38β are not encoded in the cDNAs cloned by RT-PCR.

A distinguishing feature of MAP kinases is the conserved TXY motif in the activation loop, which includes the two sites phosphorylated to activate the kinases. In the case of p38, the intervening residue X is glycine. This motif is conserved in *pmk-2* and *pmk-3*. *pmk-1* contains Q at this position. TQY does not match the motif for any characterized MAP kinase.

pmk-1, pmk-2, and pmk-3 Comprise an Operon

The *C. elegans* genome contains many genes that are predicted to fall within operons based on their distance from upstream and downstream genes. Like bacterial operons, these genes are transcribed from a promoter region that lies upstream of the first gene of the operon (14,15).

The proximity and direct orientation of *pmk-1- pmk-3* suggest that they form an operon (figure 2A). Previous work has indicated that SL2 trans-splicing is a reliable marker of operon configuration (16,17). To find out whether *pmk-1,2, and 3* form an operon, RT-PCR from whole worm RNA was performed with SL2 as the upstream primer. PCR products of *pmk-2* and *pmk-3* should be formed if SL2 trans-splicing occurs between *pmk-1* and *pmk-2*, and between *pmk-2* and *pmk-3*. As shown in figure 2B, these products are present, supporting the conclusion that the two downstream genes are trans-spliced and consistent with the hypothesis that the *pmk* genes form an operon. In some operons, the first gene is SL1 trans-spliced (16,18); however, we did not detect SL1 trans-splicing to any of the pmk genes by RT-PCR experiments (data not shown).

Promoter of PMKs is Active in the Intestine

Using GFP as a reporter, we found that the *pmk-1* promoter is active throughout the intestine. *pmk-1*: :GFP localizes to the nuclei of intestinal cells (figure 3). An identical expression pattern was found with two independent reporter constructs of *pmk-1*; one fusion construct had GFP linked to the first three exons of *pmk-1* (DA1750) and the other fused GFP to the last exon and contained 90% of the coding region of *pmk-1* (not shown). This nuclear localization was independent of growth conditions. A *pmk-2*: : GFP fusion was expressed in the same cells but was not localized to the nuclei under any tested conditions (data not shown). Because mammalian p38 responds to cellular stress, attempts to affect cellular localization of the GFP reporter included growing the worms in the presence

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environmental stresses, such as heavy metals and high concentrations of NaCl (19,20). Because *pmk-3* is the third gene of the operon, it is likely to have a similar expression pattern (15,21,22). We did not see any expression from fusion constructs for *pmk-2* and *pmk-3* in which the promoter region upstream of *pmk-1* was excluded (data not shown). These data support the idea that *pmk-2* and *pmk-3* do not have separate promoter regions, but rather are expressed from the promoter region upstream of *pmk-1*.

PMKs Phosphorylate ATF-2, and PMK-2 and PMK-3 Are Selectively Activated by MEK6

In vitro, mammalian p38 is a substrate of MEKs 3,4, and 6 in stress-responsive MAPK pathways. *In vivo*, the best evidence exists for MEK3 and MEK6. The serine/threonine kinases TAO1 and TAO2 are capable of activating these MEKs toward p38 *in vitro* and in cells (23,24,25). Using recombinant proteins purified from bacteria, we performed linked kinase assays in order to test if the *in vitro* activity of PMK-1, PMK-2, and PMK-3 is comparable to mammalian p38α. We first activated MEKs 3,4, and 6 with TAO1. All these enzymes show enhanced phosphorylation of p38α, but none show enhanced phosphorylation of PMK-1 (not shown). On the other hand, MEK6 activated PMK-2 and PMK-3. We determined that TAO1 increases MEK6 activity toward PMK-3 five-fold (figure 4), and toward PMK-2 to a reduced extent (not shown). MEKs 3 and 4 do not phosphorylate PMK-2 (not shown) or PMK-3 (figure 4). The ability of PMK-3 to phosphorylate ATF-2 is increased eight-fold by phosphorylation by MEK6 but not by MEKs 3 or 4 (figure 4). MEK6 is able to recognize a more diverse group of structures than MEKs 3 and 4 (26); thus, it is not surprising that it is the only MEK with high activity toward PMK-3. The p38 inhibitor SB203580 inhibited the ability of both PMK-2 and PMK-3 to phosphorylate ATF-2 (figure 6). PMK-1 purified from bacteria was approximately 10 kDa smaller than predicted from its amino acid sequence when immunoblotted with anti-$(His)_6$ antibody and was slightly smaller than both PMK-2 and PMK-3 as observed on immunoblots (data not shown). Because the $(His)_6$ epitope was on the N-terminus of the fusion protein, this suggested that PMK-1 was cleaved on its C-terminus. This form of PMK-1 was not a substrate of MEKs 3, 4, or 6.

PMK-1,2, and 3 are Stimulated by Osmotic Stresses in Mammalian Cells

Mammalian p38, like its yeast homolog Hog1, is activated by osmotic stresses such as sorbitol or high NaCl. We transfected epitope-tagged versions of PMK-1,2, and 3 into HEK-293 cells and stimulated the cells with 0.5 M sorbitol or 0.7 M NaCl for 15 minutes. The overexpressed proteins were immunoprecipitated and the immune-complexes were used for kinase assays (figure 5). All three PMK proteins phosphorylate ATF-2 in response to these stimuli, thus displaying the sensitivity of mammalian p38 to osmotic stress. In response to these stresses, PMK-3 exhibited much greater kinase activity than PMK-2 and PMK-1. This may be due to its higher expression level in mammalian cells and its greater recognition by mammalian kinases. Although PMK-1 and PMK-2 were not strongly activated *in vitro* by MEK6 as PMK-3 was, they do respond like p38 to a physiological stress.

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Null Mutants of pmk-1, pmk-2

Recently, the *C. elegans* knockout consortium has generated deletion mutants *pmk-1(ok169)* and *pmk-2(gk21)*. *pmk-1(ok169)* mutants have no observable growth defects or hypersensitivity to several known stresses. However, *pmk-2* null mutants arrest and die in larval stage 1 (L1). (Although the *pmk-2* strain also carries a deletion in *unc-5*, the phenotype of *unc-5* is a locomotion defect that does not cause larval arrest or premature death.) The intestinal granule content of the L1 arrested worms deviates from normal, with fewer cells appearing to contain fat under microscopic evaluation (J McKay, personal communication, and data not shown). However, the intestine appears to be developing normally at the time of arrest. However, this larval lethality was not replicated and no abnormality of the worms was noted in dsRNAi-produced knockouts of PMK-2. Therefore, the larval lethality of the strain VC36 is most likely due to a distinct mutation of this strain unrelated to PMK-2. dsRNAi experiments aimed at producing a functional knockout of *pmk-3* failed to yield worms with a discernible phenotype (not shown).

Discussion

We have identified and characterized three proteins in *C. elegans* highly similar to mammalian p38 isoforms. MAPK family members are phosphorylated by dual specificity kinases in a TXY motif in the phosphorylation lip. PMK-2 and PMK-3 contain the TGY motif characteristic of the p38 subfamily; the third homolog, PMK-1, has a TQY motif, which differs from p38, ERK (TEY), and JNK (TPY). Interestingly, the three *pmk* genes lie within an operon. Although 25% of the genome consist of operons (27), the function of operons in *C. elegans* is uncertain. In the case of the *pmk* genes, the only known mechanism for initiating transcription of *pmk-2* and *pmk-3* is the promoter upstream of *pmk-1*. This single promoter is thus responsible for the production of three proteins that share a high percentage of sequence identity. Operons may be a consequence of conservation in the relatively small genome of *C. elegans* (27). Because MEK6 did not activate all the PMKs, our *in vitro* data support the idea that PMKs are regulated by different MEK family members. If this is so, differential regulation of PMK-1,2, and 3 may allow them to have unique functions. Their linkage in an operon may allow for their coordinated and stoichiometric production in the same cells to mediate different reactions.

In unstimulated mammalian cells, many MAP kinases are found in the cytoplasm and upon stimulation, they accumulate in the nucleus. However, the subcellular localization of p38 is poorly understood. Experiments in myocytes indicate that inactive p38 localizes around the nucleus and diffusely throughout the cytoplasm and is translocated to the nucleus upon activation, after which it returns to the cytoplasm (28). On the other hand, other experiments suggest that activation does not cause nuclear redistribution (29). Other work showed that under resting conditions, p38 localizes to the nucleus where it is activated and subsequently translocates to the cytoplasm after phosphorylating its substrate (30). The constitutive localization of PMK-1 to the nucleus and PMK-2 to the cytoplasm suggests differential mechanisms of cellular localization that may be tied to their functions. Stresses such as heavy metals and high concentration of NaCl did not affect cellular localization. PMK-1

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may contain a nuclear localization signal, although it doesn't contain a good match to any known consensus nuclear localization sequence.

Without identifying upstream activators of the PMKs, we can only speculate on the function of these kinases. Since p38 has been linked to stress responses in mammalian cells, PMK-1, 2, and 3 may form part of a stress-responsive pathway in the intestine of worms. Exposure of worms to heavy metals has been shown to elicit stress responses mainly in the pharynx but also in the intestine (21). *pmk-1* mutants do not show hypersensitivity to heavy metals, such as copper and zinc.

The larval arrest/death phenotype and altered intestinal granules of the *pmk-2* deletion mutants is a provocative phenotype but is not replicated by dsRNAi experiments. At this time, we cannot attribute this lethal phenotype to the lack of *pmk-2*. The observation of abnormal intestinal granules is interesting because p38 has been linked to adipogenesis in mammalian 3T3 cells (31). There are no fat cells in *C. elegans*; rather they store fat as granules in the intestinal cells (32).

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Abbreviations

MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase
MEKK	MEK kinase
TAO	Thousand and One amino acid kinase
GFP	green fluorescent protein
ORF	open reading frame
kDa	kilodalton
PAGE	Polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
UTR	untranslated region
PCR	polymerase chain reaction
DTT	dithiothreitol
cDNA	DNA complementary to RNA
HEK	human embryonic kidney
IB	immunoblot
IP	immunoprecipitation
HSP	heat shock protein
LPS	lipopolysaccharide
TNF	tumor necrosis factor
HOG	high osmolarity glycerol
ATF-2	Activating Transcription Factor-2

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p38        1 ............................................................
PMK-1      1 ............................................................
PMK-2      1 ............................................................
PMK-3      1 MASVPSSSSLPVSHVRRHEDVSTPSAPPTKRSNNQSQPPESYEPNTWLQQQREQEQQKKL

p38        1 .............................MSLIRKKGFYKQDVNKTAWELPKTY....VS
PMK-1      1 ................MFPQTTMDHILHPTPR...EGYYVVELNRSVWVVPNYY....IN
PMK-2      1 .....MGMSATMGDSASIPGVFFADFGPAPPEITPEGYHEVELNKTKWVLPQWY....NS
PMK-3     61 AAENIKKQSIEATGNNEMVGEEEEDILSKPCGPHKRRFQFVMIRNITFAIPEGYDVEPNS

p38       28 PTHVGSGAYGSVC.SAIDKRSGEK..VAIKKPSRPFQSEIFAKRAYRELLLLKHMQHENV
PMK-1     38 ITPIGTGAYGTVC.AAECTRSGTR..VAIKKFNRPFQSIIHARRTYRELRLLRCMCHENI
PMK-2     52 LKPLGEGAYGVVC.TAEYEPTGDR..VAIKKFFRPFQSTIHAKRTYRELKLLRTLQHDNV
PMK-3    121 IEYLGGGSFGNVIKTSAVCRDGLRRYVAIKKMREPFFDPHHARRIFRETKLLQLMRHDNI

p38       85 IGLLDVFTPASSLRNFYDFYLVMPFMQTDLQKIMGME.......FSEEKIQYLVYQMLKG
PMK-1     95 IDLLDVFTPNENVNDIEDVYFVSMIMGADLSNILKIQR......LNDDHIQFLVYQILRG
PMK-2    109 LEMIDVFTPDPDASSLNNVYFVSVLMGSDLQNIMKIQR......LTDEQIQLLIYQVLRG
PMK-3    181 ICALDIYTPDEE.NDFRDVYVVTEFAGRSLYQILKQQRDYGRRVLTDEHIKFIIYQIIRA

p38      138 LKY.............................IHSAGVVHRDLKPGNLAVNEDCELK...
PMK-1    149 LKY.............................IHSADIIHRDLKPSNIAVNEDCELK...
PMK-2    163 LKYMSHQNFNSTIILKKLMHPFQRRNTRFRLYIHSAGIIHRDLKPSNIAVNERCEVKVFL
PMK-3    240 LKY.............................IHSANIIHRDLKPGNLALTDDSDLM...
                                            ***

p38      166 ..............VLDFGLARH...ADAEMTGYVVTRWYRAPEVILSWMH.YNQTVDIW
PMK-1    177 ..............ILDFGLARQ...TDSEMTGYVATRWYRAPEIMLNWMH.YTQTVDVW
PMK-2    223 SFSQLSFLILSFFKILDFGLARA...QDAEMTGYVATRWYRAPEIMLNWMH.YTQTVDVW
PMK-3    268 ..............ILDFGLARSLEKKDTSLTQYVQTRWYRSPEVIYWKIDSYTNLADMW

p38      208 SVGCIMAEMLTGKTLFRGKDYLDQLTQILKVTGVPGTEFVQKL...NDKAAKSYIQSLPQ
PMK-1    219 SVGCILAELITGKTLFPGSDHIDQLTRIMSVTGTPDEEFLKKI...SSEEARNYIRNLPK
PMK-2    279 SVGCILAELVSGRPLFPGDDHIDQLTKIMSVVGTPKEEFWSKI...QSEEARNYIKNRSP
PMK-3    314 SLGCIAAELLTGEPLFPGDEPNAQYQRITQICGSPDEELLTKIENDNSSAIKAVIQSYTT

p38      265 TPRKDFTQLFPRASPQA..ADLLEKMLELDVDKRLTAAQALTHPFFEPFRDPEEETEAQQ
PMK-1    276 MTRRDFKRLFAQATPQA..IDLLEKMLHLDPDRRPTAKEAMEHEYLAAYHDETDEPIAEE
PMK-2    336 IIRQDFVTLFPMASPYA..LELLEMMLILDPDRRISVSSALRHDYLREYSVPNDEPVAMD
PMK-3    374 HKRRNFRDVFSAHNPSEDFIDLLEKLLVLDPEKRITVEEAIQHPYLAEFSLPEDEPRADH

p38      323 PFDDSL......EHEKLTVDEWKQHIYKEIVNFSPIARKDSRRRSGMKL...........
PMK-1    334 MDLNDDV.......RADTIDEWKKIIWEEISDFQ...KNVAFADEEEDEEKMES......
PMK-2    394 TVINSIVTIDPAEERATTLSDWRELIWNEIRLFQNSARRLSFVSCTDTEEEPMKI.....
PMK-3    434 IFDLDDSQARTRFEWRGKAQRSRNRILHTCKFEKQQMQKYFITDAVWKEIMNYKRLSSSP

p38          ........
PMK-1        ........
PMK-2        ........
PMK-3    494 LIPGEADR
```

Figure 1. Sequence alignment of p38β and PMK-1,2 and 3

Sequences obtained from cloned cDNAs were aligned using multalin and boxshade [12]. PMK-3 shares the highest identity (60%) and similarity (75%) to p38β followed by PMK-2 and then PMK-1. The TXY motif is noted by asterisks.

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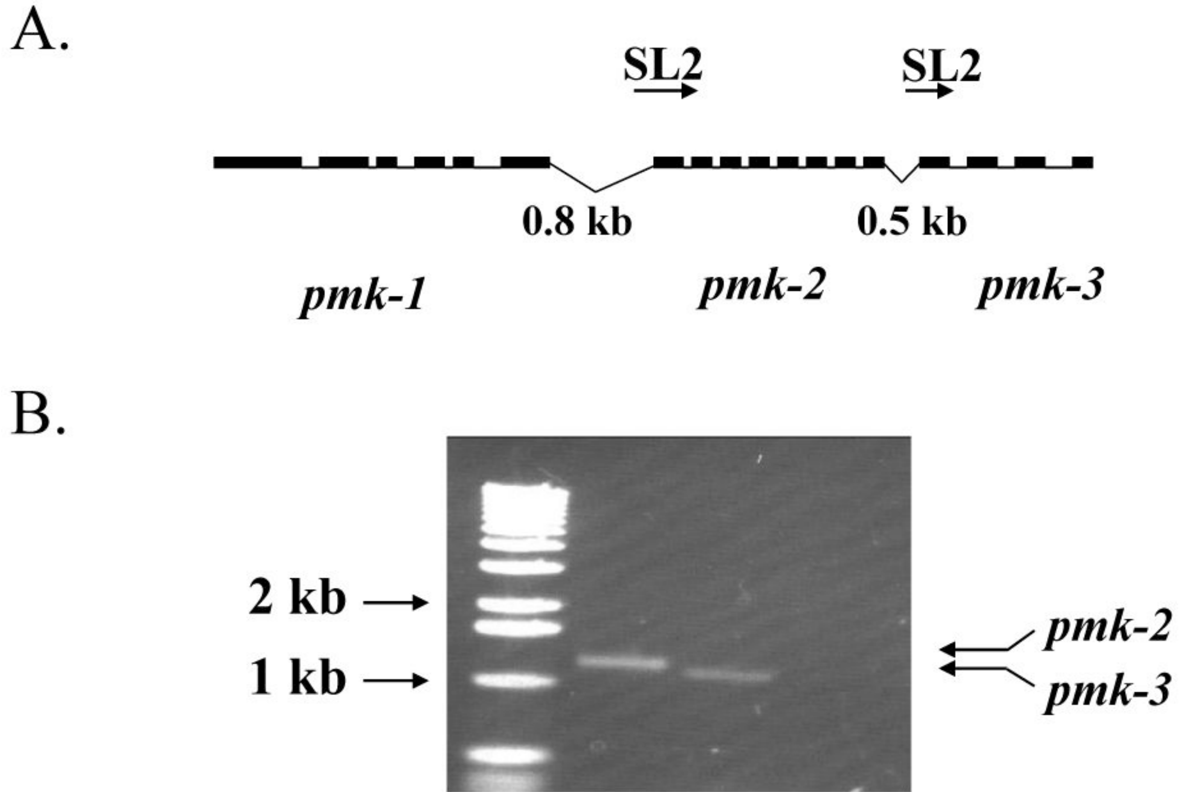

Figure 2. *pmk-2 and pmk-3* are SL2 trans-spliced

(A) A schematic of the location of the genes on chromosome IV. Boxed regions indicate exons. *pmk-1* and *pmk-2* are separated by 800 bases while 500 bases separate *pmk-2* and *pmk-3*. (B) RT-PCR was performed on whole worm RNA using an oligonucleotide with the SL2 sequence as the upstream primer and the last 20 base pairs of either *pmk-2* or *pmk-3* as the downstream oligonucleotide. Amplified products were resolved on a 1% agarose gel and stained with ethidium bromide. Lane 1 is the size marker, lane 2 is *pmk-2*, and lane 3 is *pmk-3*.

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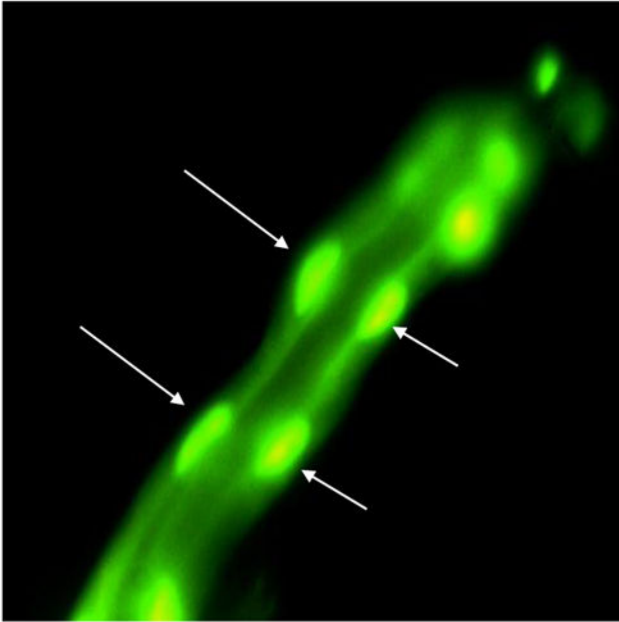
A.



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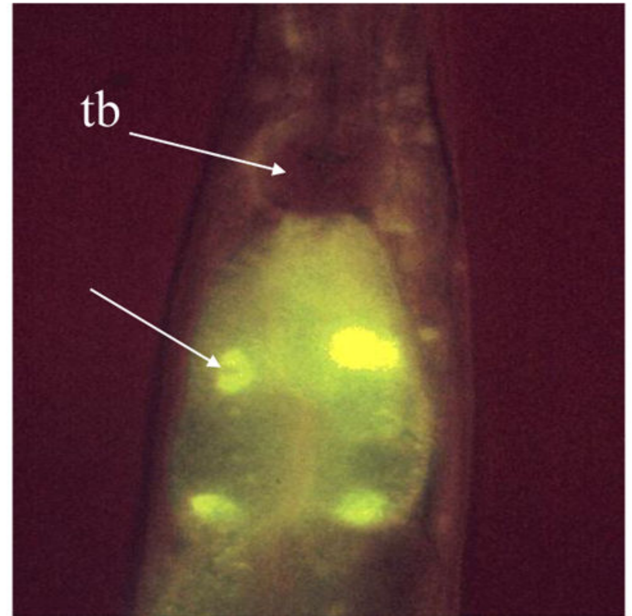

Figure 3. GFP expression directed by the *pmk-1* promoter

pmk-1: :GFP was injected into worms to determine its expression pattern. (A) Expression is seen throughout the intestine, with much of the GFP being localized to the nuclei of the intestinal cells (noted by arrows). (B) A higher magnification showing expression in the nuclei of the first four intestinal cells (one such nucleus in noted by an arrow). No promoter activity was detected in pharyngeal muscle, including the terminal bulb (tb).

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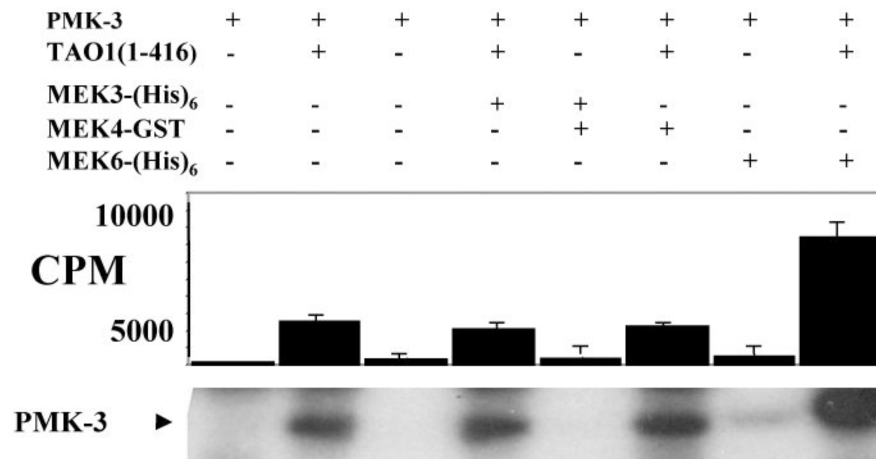
A.



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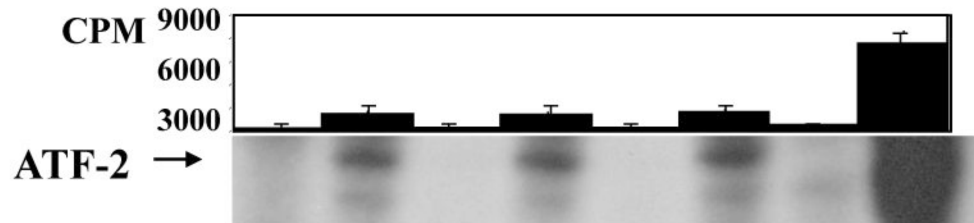

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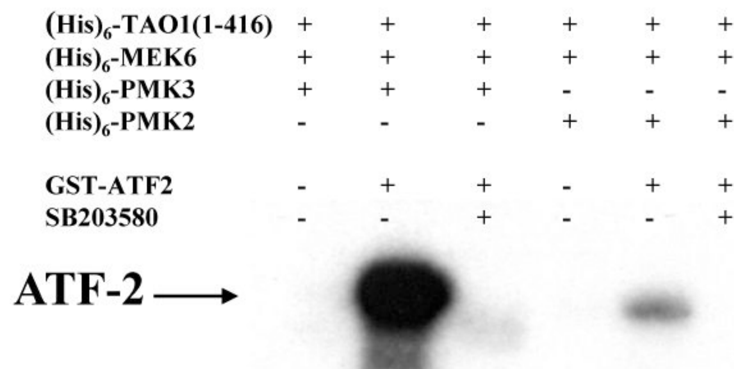

Figure 4. MEK6 phosphorylates and activates PMKs

(A) Linked kinase reactions were performed using recombinant $(His)_6$-TAO1(1-416), which was incubated alone or with recombinant MEK3, MEK4, or MEK6. A portion of this first reaction was incubated with recombinant $(His)_6$-PMK-3 and analyzed by SDS/PAGE and autoradiography. Phosphorylation of PMK-3 was quantitated using a scintillation counter. (B) A portion of the linked reactions from A were incubated with ATF-2, analyzed by SDS/PAGE and ATF-2 phosphorylation was quantitated. (C) Activated PMK-2 and PMK-3 were incubated with ATF-2, resolved on SDS/PAGE and autoradiographed. Addition of

SB203580 at 175 μM inhibited the ability of PMK-2 and PMK-3 to phosphorylate ATF-2. Note that PMK-3 has more activity than PMK-2 toward ATF-2.

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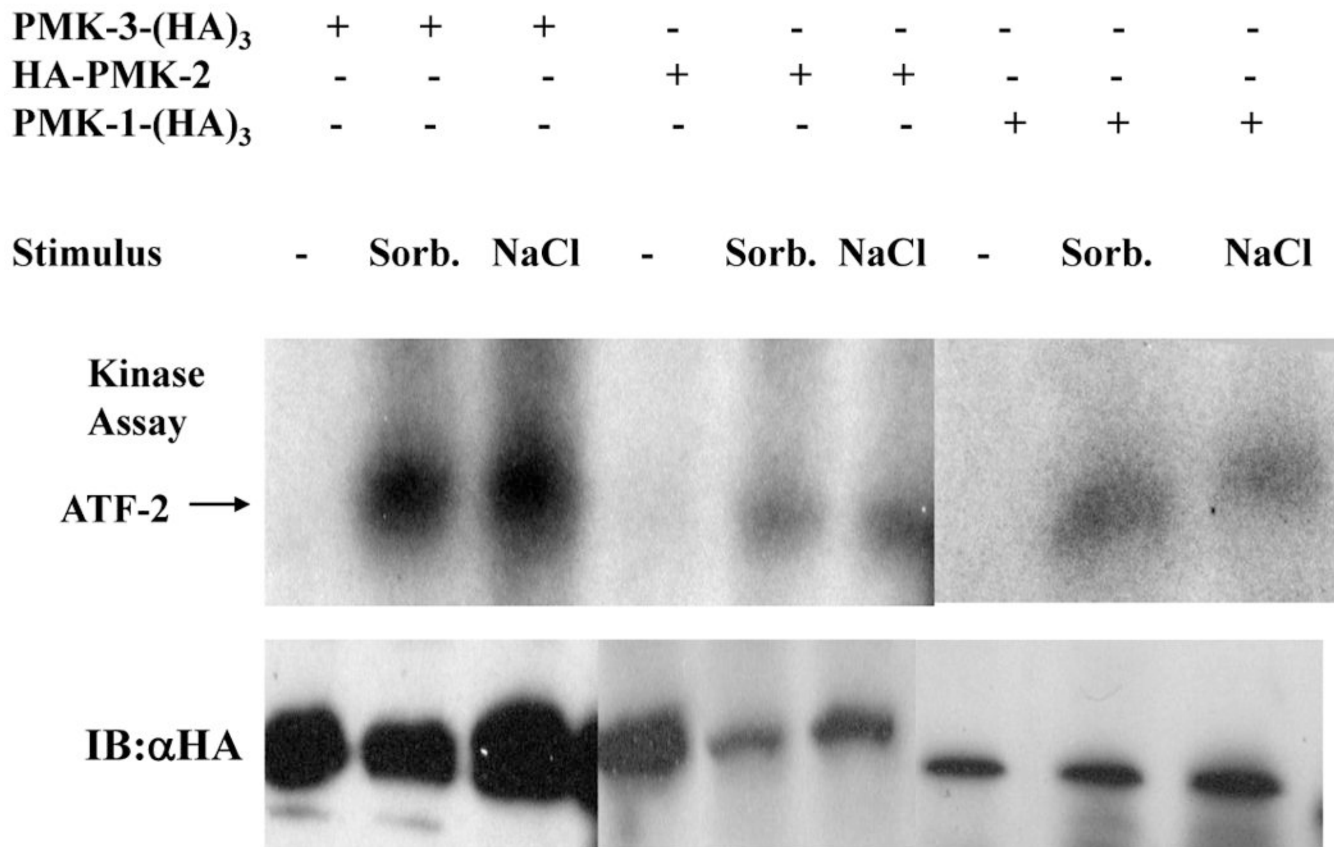

Figure 5. PMK proteins are stimulated by osmotic stresses in mammalian cells

HEK-293 cells were transfected as indicated and incubated in medium containing either 0.5 M sorbitol or 0.7 M NaCl for fifteen minutes prior to harvest. Proteins were immunoprecipitated with a monoclonal anti-HA antibody and incubated with ATF-2 in kinase assays. Reactions were analyzed by SDS/PAGE and autoradiography.

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