Cloning and Characterization of a Human STE20-like Protein Kinase with Unusual Cofactor Requirements*

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We cloned and characterized a novel human member of the STE20 serine/threonine protein kinase family named mst-3. Based on its domain structure, mst-3 belongs to the SPS1 subgroup of STE20-like proteins, which includes germinal center (GC) kinase, hematopoietic progenitor kinase (HPK), kinase homologous to STE20/SPS1 (KHS), kinases responsive to stress (KRS1/2), the mammalian STE20-like kinases (mst1/2), and the recently published STE20/oxidant stress response kinase SOK-1. mst-3 is most closely related to SOK-1, with 88% amino acid similarity in the kinase domain. The similarity of the mst-3 kinase domain to STE20 is 42%. The mst-3 transcript is ubiquitously expressed, and the protein was found in all human, mouse, and monkey cell lines tested. An in vitro kinase assay showed that mst-3 can phosphorylate basic exogenous substrates as well as itself. Interestingly, mst-3 prefers Mn2⁺ to Mg2⁺ as a divalent cation and can use both GTP and ATP as phosphate donors. Like SOK-1, mst-3 is activated by auto-phosphorylation. However, a physiological stimulus of mst-3 activity was not identified. mst-3 activity does not change upon exposure to several mitogenic and stress stimuli. Overexpression of mst-3 wild-type or kinase-dead protein affects neither the extracellular signal-regulated kinases (ERK1/2 or ERK6), c-Jun N-terminal kinase (JNK), p38, nor pp70S6 kinase, suggesting that mst-3 is part of a novel signaling pathway.

Eukaryotic cells are able to couple extracellular signals to specific biological processes such as cell growth, differentiation, and stress responses through the activation of distinct evolutionarily conserved intracellular signaling cascades, collectively known as mitogen-activated protein kinase (MAPK) cascades. In both mammals and lower eukaryotes, the core of MAP kinase cascades is a three-component module consisting of a generic MAPK kinase kinase, which phosphorylates and activates a dual-specificity MAPK kinase, which in turn activates the MAPK. In many cases the activated MAP kinases translocate to the nucleus where they phosphorylate transcription factors, thus eliciting the biological response (1). In the budding yeast Saccharomyces cerevisiae, at least six MAPK pathways have been identified. They regulate diverse biological processes such as mating and invasive growth, cell wall integrity, and the response to high osmolarity as well as pseudohyphal development and spore formation in diploid cells (2–5). As in yeast, several mammalian MAPK pathways have been identified (6, 7). The best characterized pathway is the mitogen signaling pathway, which leads to the activation of the extracellular signal-regulated kinases 1/2 (ERK1/2) through both growth factor and G protein-coupled receptors (3, 8). ERKs 1/2 are activated by the MAPK kinases, MEK1/2 (9, 10). On the level of the MAPK kinase kinase, MEK1 can be activated by several kinases, among them Raf (11), Mos (12), and to a lesser extent mitogen-activated protein kinase kinase kinase 1 (13). Other mammalian MAPK pathways can be activated by a variety of stress agents like tumor necrosis factor α, interleukin-1, UV light, and osmotic shock and lead to the activation of the MAPKs c-Jun N-terminal kinase (JNK) and p38 (14–16). Like the ERKs, JNK and p38 are activated by distinct upstream kinases. MAP kinase kinase 4 (MKK4) (17), also called stress-activated protein kinase kinase 1 (SEK) (18), activates both JNK and p38 (17), whereas MKK3 and MKK6 specifically activate p38 (17, 19–22).

Based on epistasis experiments, the S. cerevisiae serine-threonine kinase STE20 was placed upstream of the MAPK module consisting of the MAPK kinase kinase STE11, the MAPK kinase STE7, and the MAPKs FUS3/KKS1 in the mating pathway (23, 24, 25). Other S. cerevisiae STE20-like kinases are Cla4 (26), which is involved in budding and cytokinesis and SPS1 (27), a kinase required for the late events of sporulation. The mammalian STE20-related kinases represent a rapidly growing kinase family. Recently identified mammalian members of this family are the rat and mouse p21-activated protein kinases (p65PAK and mPAK-3) (28, 29) and its human counterparts, hPAK65 (hPAK2) (30, 31) and hPAK1 (32, 31), the germinal center kinase, GCK (33), and its murine equivalent, the rab8-interacting protein (rabip) (34), a hematopoietic progenitor kinase (HPK) (35), a kinase homologous to STE20/SPS1 (KHS) (36), the mammalian STE20-like kinases mst-1 (37) and mst-2 (37), kinases responsive to stress (KRS1/2) (48), and the STE20/oxidant stress response kinase-1 (SOK1) (38). Based on their structure and regulation, members of the STE20 family can be divided into two subfamilies. First, like STE20 and Cla-4, the mammalian PAKs consist of a C-terminal kinase domain and an N-terminal regulatory domain and have a small GTPase Rac1/Cdc42 binding region. PAKs bind to GTP-Cdc42 and Rac but not Rho (28–31). Upon binding, they autophosphorylate and are activated (28–31). Second, all other STE20-like kinases identified thus far re-

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The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MBP, myelin basic protein.
seem SPS1 in that they possess an N-terminal kinase domain and a C-terminal regulatory domain and do not contain a small GTPase Rac-1/Cdc42-binding domain.

Coexpression studies have demonstrated that some mammalian STE20 homologues are able to activate mammalian MAPK pathways. Activated PAKs can specifically activate the JNK and p38 pathways but not the ERK pathway. GCK, HPK, and KHS can also activate JNK but not p38 or the ERKs (35, 36, 39). These kinases do not contain the Rac1/Cdc42-binding domain, and JNK activation is thus presumed to be Rac1/Cdc42-independent.

In this paper we describe the cloning and initial characterization of a human STE20 homologue (mst-3) with unusual kinase activity characteristics. mst-3 belongs to the Sps1 subfamily of STE20-like kinases with an N-terminal kinase domain and a C-terminal regulatory domain. mst-3 mRNA and protein is ubiquitously expressed. mst-3 kinase prefers manganese to magnesium as a co-factor and can use both ATP and GTP as phosphate donors. mst-3 has high basal activity and does not activate any of the known mammalian MAPK pathways. In vitro, mst-3 kinase activity is positively regulated by autophosphorylation. Immunolocalization analysis demonstrates that mst-3 is predominantly localized in the cytoplasm.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequencing—Degenerate oligonucleotides were designed based on conserved regions of subdomains II and VII in the kinase domain STE20 and pAK. Sense (5'AG/TAC/CTAT/CA/TCA/CT/CAT/CA/CAG/ATCG/CCATCATA/CTAA/AG) and antisense (CAT/AG/TAT/ACGT/CCCGAAG/TCTCG/CA/GA/AA/CTC/GAT/CCAGA/GT/CTG) primers were used to amplify 200-nucleotide cDNA fragments from a human HeLa cell library (CLONTECH). These fragments were subcloned into pBluescript (Promega) and sequenced using the dideoxy chain termination method with Sequenase 2.0 (U. S. Biochemical Corp.). Sequence was analyzed using DNASTAR. Data base searches were done using BLAST. Several clones encoded kinase domain fragments of novel kinases related to the yeast STE20 and p65PAK were used to amplify a 200-nucleotide fragment from a HeLa cell cDNA library. This probe was generated and used to screen 10^6 plaques of a random-primed human oligo(dT)-primed human HeLa cell library (courtesy of Dr. R. Reed). Three plaques with identical sequence were purified and isolated; one clone contained SPS1 in that they possess an N-terminal kinase domain and a C-terminal regulatory domain and do not contain a small GTPase Rac-1/Cdc42-binding domain.

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probe (encompassing nucleotides 1–350) was generated and used to screen a random-primed HeLa cell library. One clone was isolated that contained a stop codon upstream of the putative start codon and no other candidate initiation start codon in between. However, no putative polyadenylation site was identified (Fig. 1).

Amino Acid Sequence Analysis and Similarity to Other Kinases—The 2.0-kilobase cDNA contains an open reading frame of 431 amino acids with a predicted molecular mass of 48 kDa (Fig. 1). mst-3 contains all subdomains of serine/threonine kinases (40). Using the BLAST program to determine sequence homologies to other kinases, mst-3 was identified as a member of the STE20 family of protein kinases. mst-3 consists of an N-terminal kinase domain and a 142-amino acid C-terminal regulatory domain. The overall structure of mst-3 more closely resembles the SPS-1 group of the STE20 family of protein kinases (see Fig. 2C). mst-3 is highly related to the recently published SOK-1 kinase (38), with 88% identity in the kinase domain and 68.8% overall amino acid identity. The mst-3 kinase domain is related to the kinase domains of mst-1/KRS1 (37, 48), mst-2/KRS2 (41, 48), GCK (33), HPK (35), hPAK65 (30), and STE20 (23, 24) with 56, 56, 48, 44, 48, 45, 42% amino acid similarities, respectively (Fig. 2B). The mst-3 C-terminal regulatory domain does not contain a Cdc42/Rac binding region and does not have any identifiable sequence motifs other than several acidic regions.

Analysis of mRNA and Protein Expression—Northern blot analysis using poly(A) RNA from multiple human tissues demonstrated that the mst-3 transcript is ubiquitously expressed (Fig. 3). Under high stringency conditions, using a unique region upstream of the kinase domain as a probe, a single 2-kilobase mRNA was detected that is expressed at highest levels in heart, skeletal muscle, and pancreas. To confirm equal loading of mRNAs, the blot was re-probed using a human β-actin probe (data not shown).

The expression of mst-3 protein was examined using Western blot analysis. A polyclonal antiserum (a mst-3) raised against a glutathione S-transferase fusion of the full-length mst-3 protein recognized a protein of about 52 kDa in all human (Hela, C2C12, 293, A431), mouse (NIH3T3), and monkey cells (COS) tested (Fig. 4A). The increased apparent molecular mass could be due to posttranslational modifications. Using either preimmune serum or antibody preadsorbed with antigen abolished the signal, demonstrating the specificity of the a mst-3 serum (data not shown). An HA-tagged recombinant mst-3 (HAmst-3) that contained a 26-amino acid extension at the N terminus could be detected as a slower migrating band on Western blots when transfected into COS cells (Fig. 4A). a mst-3 also immunoprecipitated a single protein of the same size from 35S-labeled COS cell lysates under denaturing conditions (Fig. 4B, lane 2) that was absent from immunoprecipitates performed using identical conditions but with either preimmune serum (Fig. 4B, lane 1) or antigen-preadsorbed omst-3 antiserum (Fig. 4B, lane 3) for the immunoprecipitation.

Catalytic Activity of mst-3—omst-3 was used to immunoprecipitate endogenous mst-3 from COS lysates to develop an in vitro kinase assay. No phosphotransferase activity above the
**Fig. 2.** Sequence comparison of mst-3 with members of the STE20 protein kinase family. A, alignment of the predicted amino acid sequence of the mst-3 kinase domain with the corresponding domains of SOK-1, mst-1, GCK, HPK, SPS1, hPAK65, and STE20. Amino acids conserved in all proteins are boxed. B, a schematic showing percent amino acid similarities of the kinase domains members of the STE20 protein kinase family. C, this schematic shows the structures of mst-3 and related kinases. The kinase domains are drawn as hatched boxes. p21 binding regions are indicated as solid bars.
Autophosphorylation and Activation of mst-3—As described above (Fig. 5, A and C), a phosphoprotein of the size of mst-3 appeared after in vitro kinase assays were performed in the presence of Mn$^{2+}$ (Fig. 5E). The autoradiographs shown in Fig. 5E were representative of at least two experiments. KD, kilodaltons.

control preimmune precipitates was detected using histones H1, H2, H4, casein, and phosphitin as substrates (data not shown). However, mst-3 readily phosphorylated MBP and histone H3 at levels significantly above control (Fig. 5A). A phosphoprotein migrating at the size of mst-3 was also detected, suggesting that mst-3 autophosphorylates (Fig. 5A, lanes 2 and 4). Although the presence of a protein in the immunocomplex that phosphorylates mst-3 cannot be excluded, it is unlikely since the phosphoprotein was still present after immunoprecipitation and visualized by autoradiography. The results are representative of at least two experiments.

To determine the mst-3 cofactor requirements, mst-3 activity was examined using either Mn$^{2+}$ or Mg$^{2+}$ in the in vitro kinase assay. HA-tagged wild-type (HAMst-3wt) or HAMst-3KR was transfected into COS cells, and lysates were immunoprecipitated using either preimmune serum (Fig. 5B, lanes 1, 3, 5, and 7) or a polyclonal HA antibody (Fig. 5B, lanes 2, 4, 6, and 8). Untransfected COS cell lysates were precipitated either with preimmune serum (Fig. 5B, lanes 10 and 12), or mst-3 serum (Fig. 5B, lanes 11 and 13), or with antigen-preadsorbed mst-3 (Fig. 5B, lane 9) to precipitate endogenous mst-3. In vitro kinase assays were then performed in the presence of 10 mM MgCl_2 or 5 mM MnCl_2 and 1 mM ATP using histone H3 as a substrate. Interestingly, under these conditions, mst-3 catalytic activity was consistently found to be 20–50-fold higher in the presence of Mn$^{2+}$ compared with Mg$^{2+}$. Similarly, the autokinase activity in the presence of manganese was elevated (Fig. 5C, compare lane 2 with lane 4). No activity above background can be immunoprecipitated from lysates expressing HAMst-3KR, suggesting that the observed activity is not due to coinmunoprecipitated enzymes (Fig. 5C, lanes 6 and 8).

The intracellular concentration of Mn$^{2+}$ is in the μM range, whereas the Mg$^{2+}$ concentration is in the mM range (42). To determine whether Mn$^{2+}$ is able to activate mst-3 at physiological concentrations, mst-3 activity was tested using a range of Mg$^{2+}$ and Mn$^{2+}$ concentrations (Fig. 5, D and E). Endogenous mst-3 was immunoprecipitated from HeLa cells, and in vitro kinase assays were performed using the indicated Mn$^{2+}$ and Mg$^{2+}$ concentrations. Mn$^{2+}$ at μM concentrations could activate mst-3 to higher levels than Mg$^{2+}$ at mM concentrations (compare mst-3 activity at 100 μM Mn$^{2+}$ to 10 mM Mg$^{2+}$). The minimum Mn$^{2+}$ concentration at which a small increase in mst-3 activity was seen, was 50 μM (Fig. 5D). Optimal activity was achieved at 1 mM. Above 5 mM, Mn$^{2+}$ was inhibiting, and mst-3 activity decreased. Mg$^{2+}$ did not activate mst-3 at μM concentrations (Fig. 5D). Optimal Mg$^{2+}$-regulated mst-3 activity was observed with 10 mM Mg$^{2+}$, and 20 mM was not inhibiting (Fig. 5E). These results suggested that Mn$^{2+}$ may play a role in mst-3 regulation under physiological conditions.

Phosphoamino acid analysis of in vitro phosphorylated MBP and histone H3 revealed that mst-3 is a serine/threonine protein kinase. Mst-3 phosphorylated MBP on Ser and Thr and histone H3 on Thr. Mst-3 also autophosphorylated on threonine (Fig. 5F).
shown), suggesting that although mst-3 had high basal activity, it was not constitutively active.

Mst-3 activity was also elevated 1.5–3-fold by incubating cells with calyculin A, a potent inhibitor of type 1c and 2a serine/threonine phosphatases but not by incubation with vanadate, a general tyrosine phosphatase inhibitor (Fig. 7B), suggesting that mst-3 is only partially regulated by phosphorylation.

Regulation of mst-3 Kinase Activity—To determine the position of mst-3 in signaling pathways, we coexpressed HA\textit{mst-3wt} and HA\textit{mst-3KR} with several MAP kinases (ERK-1, JNK, p38, ERK-6) and pp70-S6 kinase. In unstimulated versus stimulated cells, neither HA\textit{mst-3wt} nor HA\textit{mst-3KR} activated or inhibited ERK-1, JNK, p38, ERK-6, and pp70-S6 kinase.²

Furthermore, we tested numerous agonists representing different classes of stimuli for their ability to activate or inhibit mst-3 activity in various cell types (Table I). In view of the high basal activity of mst-3, all time course assays were done in parallel with assays to ensure linear kinase assay conditions. No activating or inhibitory stimuli were found. Surprisingly, inducers of oxidative stress (H\textsubscript{2}O\textsubscript{2} and menadione), which have been shown to activate the closely related SOK kinase (38), did not activate mst-3.

² K. Schinkmann, unpublished information.
Subcellular Distribution of mst-3—To determine the subcellular distribution of mst-3, NIH3T3 fibroblasts were transiently transfected with HAmst-3wt, and the expressed protein was visualized using a HA monoclonal antibody. Nuclei were visualized with Hoechst (Fig. 8, B and D). HAmst-3wt was localized predominantly in the cytoplasm (Fig. 8A). No staining was visible in the control untransfected cells (for an example see Fig. 8A) or HAmst-3-transfected cells stained with secondary antibody only (Fig. 8C).

**DISCUSSION**

We have cloned and characterized a novel human member of the growing family of STE20-related protein kinases. Pending the identification of a physiological function, this kinase was named mst-3. Based on its structure, mst-3 belongs to the SPS1-like subfamily of STE20-like kinases with an N-terminal catalytic and a C-terminal regulatory domain. mst-3 is most closely related to the recently identified SOK-1 kinase (38) with amino acid similarities of 88 and 68.8% in the kinase domain and overall protein sequence, respectively. However, mst-3 is not regulated in the same fashion as SOK-1 and exhibits distinct kinase activity characteristics.

Western blot analysis of mst-3 shows that it is expressed ubiquitously in all cell lines tested, including human (A431, 293, C2C12), monkey (COS), and mouse (NIH3T3). An *in vitro* kinase assay using HA-tagged recombinant as well as endogenous mst-3 was developed and revealed that mst-3 possesses unusual cofactor requirements. The ability to phosphorylate itself and exogenous substrates is consistently 20–50-fold higher in the presence of 1 μM ATP versus 1 μM GTP in the presence of 5 mM manganese. The results shown are representative of at least four experiments.

**FIG. 6.** Comparison of GTP and ATP as phosphate donors. A, endogenous mst-3 was immunoprecipitated and subjected to immunocomplex kinase assays as described in the legend to Fig. 5 in the presence of 5 mM manganese, 1 mM ATP, and the indicated concentrations of GTP. B and C, immunocomplex kinase assays of endogenous mst-3 (B) or HAmst-3-wt or HAmst-3-KR (C) were performed in the presence of either 1 μM ATP or 1 μM GTP in the presence of 5 mM manganese. The results shown are representative of three experiments.

**FIG. 7.** Activation of mst-3 activity by autophosphorylation (A) and by phosphorylation (B). A, endogenous mst-3 was immunoprecipitated from growing HeLa cells and subjected to immunocomplex kinase assays. Lysates were preincubated (preinc.) in the presence of 1 μM ATP for 20 min or not followed by a 3-min incubation with histone H3. Activity toward the substrate was analyzed as described in the legend of Fig. 5. The depicted result is representative of at least six experiments. B, growing HeLa cells were treated with calyculin A (10 nM, 30 min, lane 2) or vanadate (0.1 mM, 30 min, lane 3) or left untreated (lane 1). Immunocomplex kinase assays were performed as described in the legend of Fig. 5. The experiment shown is representative of at least four experiments.
mst-3, a Mn$^{2+}$-regulated STE20-like Protein Kinase

To examine the role of Mn$^{2+}$ versus Mg$^{2+}$, we studied the effect of different Mn$^{2+}$ and Mg$^{2+}$ concentrations on mst-3 activation. Mn$^{2+}$ within the concentration interval of 0.05–5 mM stimulates mst-3 activity (Fig. 5D), whereas higher concentrations inhibit it (Fig. 5E). At a concentration of 0.5 mM Mn$^{2+}$ and 50 mM ATP, 97–98% of the ATP is present as MnATP, and increasing the Mn$^{2+}$ concentration does not lead to a significant MnATP increase (43). The increase of mst-3 activity observed in response to increasing Mn$^{2+}$ concentrations (Fig. 5D and E) thus suggests that besides formation of the substrate MnATP, binding of free Mn$^{2+}$ to a distinct site on mst-3 may be required for full kinase activation. The inhibitory effect of Mn$^{2+}$ concentration above 5 mM (Fig. 5E) might be due to an inhibitory feedback mechanism.

Under physiological conditions, the concentration of Mn$^{2+}$ is in the μM range, about 3 orders of magnitude below the intracellular Mg$^{2+}$ concentration, which, like the ATP levels, lies in the mM range. The fact that the Mn$^{2+}$ concentration required for mst-3 activation was about 2 orders of magnitude lower than the Mg$^{2+}$ concentration at the μM ATP levels used in our assays combined with the observation that Mn$^{2+}$ could activate mst-3 to much higher levels than Mg$^{2+}$ suggested that Mn$^{2+}$ is a physiological regulator of mst-3. However, the physiological role of Mn$^{2+}$ in mst-3 activation is unclear.

Subdomains VI and VIII of protein kinases contain residues that are conserved among members of either the serine/threonine kinases or the tyrosine kinases (40). The most striking indicator of amino acid specificity lies in subdomain VI. Tyrosine kinases contain either D/EARK (src family) and D/EARLN (all others), whereas serine/threonine kinases contain DLPKEN. In mst-3, this indicator region resembles more the tyrosine kinases (D/EAKAN), whereas the region in subdomain VIII classifies it as a serine/threonine kinase GTPFWMAPE (40). Phosphoamino acid analysis demonstrates that mst-3 is a serine/threonine kinase (Fig. 5P).

To place mst-3 into one of the existing signaling pathways or a novel pathway, we attempted to stimulate or inhibit its activity using several mitogenic and stress stimuli (Table I). Due to the high basal activity of immunoprecipitated endogenous and recombinant mst-3, immunocomplex kinase assays performed under standard kinase assay conditions would not be in the linear range and would thus mask activation. Therefore, all stimulation experiments were performed under optimized kinase assay conditions. However, no consistent stimulus of mst-3 activity was identified. Mst-3 activity does not change upon growth arrest due to serum deprivation or growth promoting agents such as serum, lysophosphatidic acid, epidermal growth factor, platelet-derived growth factor, and phorbol 12-myristate 13-acetate. The stress stimuli anisomycin, sorbitol, staurosporine, and UV exposure did not increase mst-3 activity in the indicated cell lines (Table I). Unexpectedly, mst-3 could not be further activated by oxidative stress due to H$_2$O$_2$ or menadione, which have been reported to activate the closely related SOK-1 kinase (38). One explanation for this discrepancy is that mst-3 and SOK-1 have distinct biochemical properties, that Mn$^{2+}$ is not a cofactor for SOK-1, and that despite their closely related sequence, mst-3 and SOK-1 are regulated differentially and are part of separate signaling pathways.

mst-3 activity does not change upon raising the intracellular Ca$^{2+}$ or cAMP concentrations. We also tested serine/threonine and tyrosine phosphatase inhibitors on mst-3 activity, achieving partial activation of mst-3 activity with calyculin A, suggesting that full mst-3 activation might also be regulated through other mechanisms, i.e. dimerization and phosphorylation. Preliminary data indicated that mst-3 can dimerize (data not shown). In this respect mst-3 resembles mst-1, which has been shown to dimerize (46).

mst-3 wild-type or kinaseinactive protein expression did not activate or inhibit any of the four MAPK cascades (ERK-1, JNK, p38, ERK-6) nor pp70-S6 kinase in coexpression experiments (data not shown), suggesting that mst-3 is part of a novel signaling pathway. Alternatively, mst-3 activity might be necessary but not sufficient for the activation of the pathways tested.

mst-3 is not a constitutively active kinase, since immunoprecipitated mst-3 autophosphorylates and, like SOK-1 and PAK, is activated by autophosphorylation. Depending on cell type and the substrate used, a 20-min incubation in the presence of ATP without substrate followed by incubation with substrate results in a 2–8-fold increase in mst-3 activity. Thus, even though mst-3 possesses high basal activity, it is not a constitutively active kinase. At this point the physiological role of mst-3 is unknown as is the significance of its similarity to STE20. Elucidation of these critical problems will have to await the identification of physiological substrates and effectors.

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| Table I: Regulation of mst-3 activity in various cell types |
|---------------------------------------------|
| **Stimulus**           | **Cell type**          |
| Serum-starved growth factor | COS, 293, A431, NIH3T3, Swiss3T3, HeLa |
| Platelet-derived growth factor | Swiss3T3 |
| Epidermal growth factor | Swiss3T3 |
| Serum | Hela |
| Nerve growth factor | PC12 |
| Lysophosphatidic acid | Swiss3T3 |
| Phorbol 12-myristate 13-acetate | Swiss3T3 |
| Anisomycin, Hela |
| Sorbitol, Staurosporine, Menadione | Swiss3T3 |
| H$_2$O$_2$, Fibronectin | Swiss3T3 |
| A23187, Forskolin | HeLa, Jurkat |

**Fig. 8. Subcellular localization of mst-3.** HA-tagged mst-3 cDNA was transfected into HeLa cells, and HA-mst-3 was visualized using αHA as a primary antibody followed by cy3 (goat anti-mouse conjugated rhodamine) as a secondary antibody (A) or cy3 only (C) as described under "Experimental Procedures." Nuclei were visualized using Hoechst staining (B and D). HA-mst-3 was localized predominantly in the cytoplasm (A).
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