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Membrane Localization of the MAK-V Protein Kinase

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Abstract—Activities of many proteins including protein kinases are often regulated by their dynamic association with specific intracellular compartments. MAK-V is an AMPK-like protein kinase with poorly characterized functions and mechanisms of action. Similarly to many other protein kinases, association of MAK-V with specific intracellular compartments could be essential for its proper functions. In this work, we studied subcellular distribution of exogenously produced and endogenous MAK-V proteins in mammalian cells using biochemical cell fractioning aiming to supplement data on MAK-V intracellular localization studied by immunocytochemical methods. We found that a significant portion of MAK-V protein in mammalian cells is associated with membranes. Moreover, MAK-V expressed in yeast was also targeted to membrane, thus suggesting an evolutionarily conservative mechanism of MAK-V membrane association. Based on the ability of various MAK-V deletion mutants to localize to membrane and comparison of MAK-V amino acid sequences from different species, we suggest a possible mechanism governing MAK-V association with intracellular membranes.

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MAK-V protein kinase is the only member of a separate subgroup within the AMPK group of protein kinases [1]. While other AMPK-related protein kinases are well conserved in evolutional context, MAK-V orthologs present only in genomes of chordates [2] and sea urchin [3], and are absent from genomes of *Drosophila*, nematode, and yeast. MAK-V protein kinase was originally identified in a result of comparative analyses of tumors with different properties [4-6]. Based on the *mak-v* expression pattern, its specific role in developmental processes and nervous system functioning was suggested [5-7], although mechanisms of MAK-V action, its targets, and molecular processes regulated by this protein kinase remain mostly unknown.

Localization in a specific intracellular compartment often is an important factor in regulation of protein functions. Our previous studies demonstrated that exogenously produced MAK-V protein kinase in COS-1 cells is specifically associated with centrosomes and, along with cytoplasmic localization, is targeted to the nucleus [8]. The presence of MAK-V on the centrosome, which plays a key role in cell division, led to the suggestion that MAK-V might play a role in cell cycle progression. Indeed, this suggestion was recently supported by Sakai et al. who found that MAK-V modulates proliferative activity of cells [9]. However, the data on MAK-V intracellular localization are far from being comprehensive. Because of the lack of antibodies able to detect MAK-V at the endogenous level in Western blot analysis and in immunocytochemical applications, localization of only exogenously produced protein has been investigated. It is known that this approach might lead to artifactual results due to high level of exogenous protein production, which is further aggravated by the tendency of MAK-V to form aggregates under conditions of transient expression. Also, only immunofluorescent analysis was used to characterize intracellular localization of MAK-V, but the cell fixation procedure itself could result in artifactual protein localization. Therefore, to comprehensively characterize MAK-V intracellular distribution, it is necessary to combine different experimental approaches as well as to assess localization of endogenous MAK-V protein. In this work, we applied various experimental approaches including analysis of intracellular distribution of endogenously produced protein and found that MAK-V protein in a cell is partially localized on membranes. We also conducted primary analysis to identify determinants in MAK-V protein mediating this localization.

Abbreviations: PI(4,5)P_2) phosphatidylinositol-(4,5)-bisphosphate; PNS) postnuclear supernatant; UBA) ubiquitin-associated domain.

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MATERIALS AND METHODS

Cell lines. CSML-0, CSML-100, VMR-0, and VMR-Liv cell lines [10] were cultured on DMEM medium (Invitrogen, USA) with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. PC12TetOn cells with inducible expression of MAK-V protein with C-terminal FLAG epitope [11] were cultured on DMEM medium with 10% heat-inactivated horse serum (Invitrogen), 5% tetracycline-screened fetal bovine serum (HyClone, USA), 100 μg/ml G418 (Calbiochem, USA), and 100 μg/ml hygromycin B (Invitrogen). To induce MAK-V-FLAG protein production, cells were treated with 1 μg/ml of doxycycline for 2 days.

Cell fractioning. Membrane and cytosolic fractions were prepared as described [12]. Briefly, cells were mechanically disrupted by passing a cell suspension through a 27G needle. After that, the homogenate was cleared from nuclei and cell debris by centrifugation. The resulting postnuclear supernatant (PNS) was separated to membrane (pellet) and cytosolic (supernatant) fractions by centrifugation (100,000*g*, 1 h).

Generation and purification of anti-MAK-V antibodies. Full-length MAK-V protein with C-terminal FLAG tag was produced in *Escherichia coli* as a chimera with glutathione-S-transferase and sequentially purified on glutathione-Sepharose (GE Healthcare, UK) and anti-FLAG-M2 affinity gel (Sigma, USA) accordingly to the manufacturer's instructions. Purified protein was used to immunize rats. Antibodies against MAK-V were affinity purified from the antiserum as described before [13]. The MAK-V-FLAG protein purified from PC12TetOn cells [11] and immobilized on Hybond-P membrane (GE Healthcare) by protein transfer from SDS-polyacrylamide gel was used as an affinity matrix for purification of antibodies, which were used for Western blotting at 1 : 20 dilution.

Western blot analysis. Proteins were separated by SDS-PAGE and transferred onto Hybond-P membrane by the semi-dry transfer method. Western blot analysis was done with primary monoclonal anti-gm130 (Transduction Laboratories, USA), anti-α-tubulin (Sigma), polyclonal anti-FLAG (Sigma), anti-Nedd4 (Abcam, UK), anti-mTOR (Cell Signaling, USA), and affinity purified anti-MAK-V antibodies and appropriate secondary antibodies conjugated with horseradish peroxidase (GE Healthcare). ECL+ reagent was used for detection (GE Healthcare).

Analysis of membrane localization in yeast. For analysis of membrane localization of proteins in yeast, cdc25H yeast strain (a component of the CytoTrap system) (Stratagene, USA) was used. To produce in yeast MAK-V protein kinase and its deletion mutants fused to hSOS protein, respective *mak-v* cDNA fragments were cloned in pSOS vector (Stratagene) in-frame with hSOS cDNA. To express in yeast MAK-V protein alone, consensus yeast translation initiation site was inserted into *mak-v* cDNA, and the resulting cDNA encoding full-length MAK-V protein was cloned into pSOS vector instead of hSOS cDNA. All manipulations with yeast were performed as recommended by the CytoTrap system manufacturer. For analysis of complementation of temperature-sensitive mutation, three independent yeast colonies carrying pSOS-based plasmid were plated onto two plates with synthetic medium lacking leucine, and the yeast were grown for two days at 25°C or for four days at 37°C.

RESULTS

Biochemical fractioning of cells with inducible MAK-V expression. PC12TetOn cells with inducible expression of MAK-V protein with C-terminal FLAG tag were used to prepare cytosolic and membrane fractions. Analysis of fractions with anti-FLAG antibodies revealed that the membrane fraction contains a significant portion of the MAK-V cellular pool (Fig. 1). To confirm correct distribution of cytosolic and membrane-associated proteins in prepared fractions, they were analyzed for the presence of α-tubulin, mTOR, Nedd4, and gm130 proteins. The analysis showed that α-tubulin, which solubilizes due to microtubule depolymerization during fractioning, was present only in cytosolic fraction. Transmembrane protein of Golgi apparatus, gm130, was found only in membrane fraction. mTOR and Nedd4 proteins, which are known to be partially associated with membranes [14, 15], were detected in both cytosolic and membrane fractions (Fig. 1). Thus the fractioning method we used has not interfered with distribution of proteins with known membrane or cytosolic localization. This allows us to conclude that exogenously produced MAK-V protein kinase is associated with membranes.

Endogenous MAK-V protein is associated with membranes. We found that exogenously produced in PC12TetOn cells MAK-V protein is associated with membranes. However, in spite of the lack of aggregated MAK-V-FLAG protein in PC12TetOn cells [11], abnormal presence of the protein in membrane fraction due to its high expression level could not be excluded. Thus we attempted to assess if endogenous MAK-V protein is associated with cellular membranes. As available affinity purified on synthetic peptide anti-MAK-V antibodies [13] failed to detect MAK-V at the endogenous level, we generated a different preparation of anti-MAK-V antibodies. We used full-length MAK-V protein as an immunogen and affinity purified antibodies on MAK-V-FLAG protein, which was isolated from PC12TetOn cells and immobilized on PVDF membrane. These antibodies were able to efficiently recognize MAK-V protein with C-terminal FLAG epitope (data not shown). To determine if the antibodies obtained are suitable for detection of

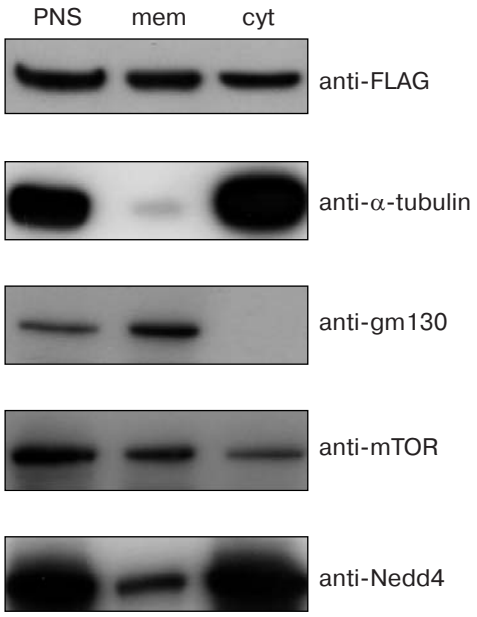

Fig. 1. Exogenously produced in PC12TetOn cells protein kinase MAK-V is associated with membrane fraction. Western blot analysis with anti-FLAG antibodies of cytosolic (cyt) and membrane (mem) fractions derived from postnuclear supernatant (PNS) of PC12TetOn cells producing MAK-V-FLAG protein. Membrane was also stained with antibodies against transmembrane gm130, cytosolic α-tubulin, and partially associated with membranes Nedd4 and mTOR proteins.

MAK-V protein at the endogenous level and to evaluate their specificity, we used lysates prepared from CSML-0 cells having high level of the *mak-v* transcript, and from CSML-100 cells, which do not express *mak-v*. On Western blotting, antibodies detected in CSML-0 cell lysate an 80 kD protein matching by mobility the MAK-V-FLAG protein, while this protein was absent from CSML-100 cells which are *mak-v*-negative (Fig. 2). The same protein was detected in lysate of VMR-Liv cells, which also express *mak-v* although at significantly lower level than CSML-0 cells, and absent from lysate of *mak-v*-negative VMR-0 cells (Fig. 2). Thus we concluded that the antibodies obtained can specifically recognize endogenously produced MAK-V protein.

Next, we fractioned CSML-0 cells and analyzed MAK-V protein distribution using the generated antibodies. Similarly to exogenously produced in PC12TetOn cell protein, endogenous MAK-V protein was predominantly found in the membrane fraction (Fig. 2). This result confirms our suggestion on association of the MAK-V protein kinase with membranes, which was made based on distribution of exogenously produced protein.

Membrane targeting of MAK-V in yeast. To further confirm that MAK-V is a membrane-associated protein, we used yeast-based biological assay for protein localization on membrane. The assay is based on complementation of temperature-sensitive mutation in cdc25H yeast. The complementation occurs when human hSOS protein is targeted to membrane by the protein of interest expressed as a chimera with hSOS, thus establishing the ability of the studied protein to localize on cellular membranes (Fig. 3a). When hSOS-MAK-V fusion protein but not hSOS alone was expressed in yeast, complementation of the mutation was observed (Fig. 3c). At the same time, expression of MAK-V protein alone had no effect. Thus MAK-V protein kinase itself is not able to complement the mutation, and mutation complementation in yeast expressing hSOS-MAK-V protein is due to membrane localization of MAK-V.

Besides the N-terminal catalytic domain, MAK-V has a typical for many other AMPK-related protein kinases ubiquitin-associated (UBA) domain located shortly after the catalytic domain as well as a unique C-terminal region (Fig. 3b). To determine roles of these structural domains in membrane localization of MAK-V protein, we analyzed abilities of hSOS protein fused to various MAK-V deletion mutants to complement temperature-sensitive mutation in yeast. Deletion of C-terminal region of MAK-V or 26 N-terminal amino acids had no effect on the ability of chimeras with hSOS to complement the mutation (Fig. 3c), thus suggesting that MAK-V membrane association is mediated by the region encompassing catalytic and UBA domains. Indeed, deletion of these domains led to inability of yeast to grow at the non-permissive temperature. Finally, deletion of a single UBA domain, although it did not lead to complete growth inhibition, resulted in significantly compromised growth under non-permissive conditions compared to full-length MAK-V protein fused to hSOS (Fig. 3c). This result demonstrates that UBA domain is necessary for efficient membrane localization of the MAK-V protein kinase.

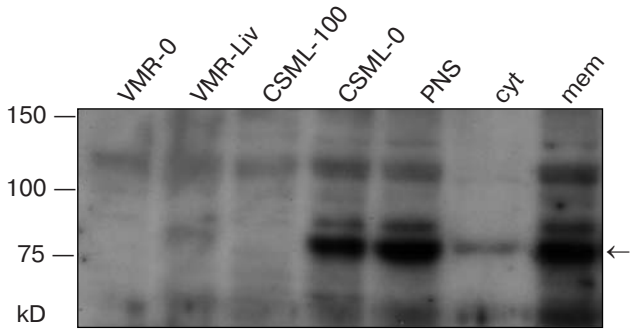

Fig. 2. MAK-V protein kinase is associated with membranes in CSML-0 cells. Lysates of CSML-0 and VMR-Liv cells expressing *mak-v*, and lysates of CSML-100 and VMR-0 cells lacking *mak-v*, as well as CSML-0 cell postnuclear supernatant (PNS) and prepared from it membrane (mem) and cytosolic (cyt) fractions were analyzed by Western blotting with anti-MAK-V antibodies. Positions of protein molecular weight markers in kD are shown on the left, and endogenous MAK-V protein is marked by an arrow.

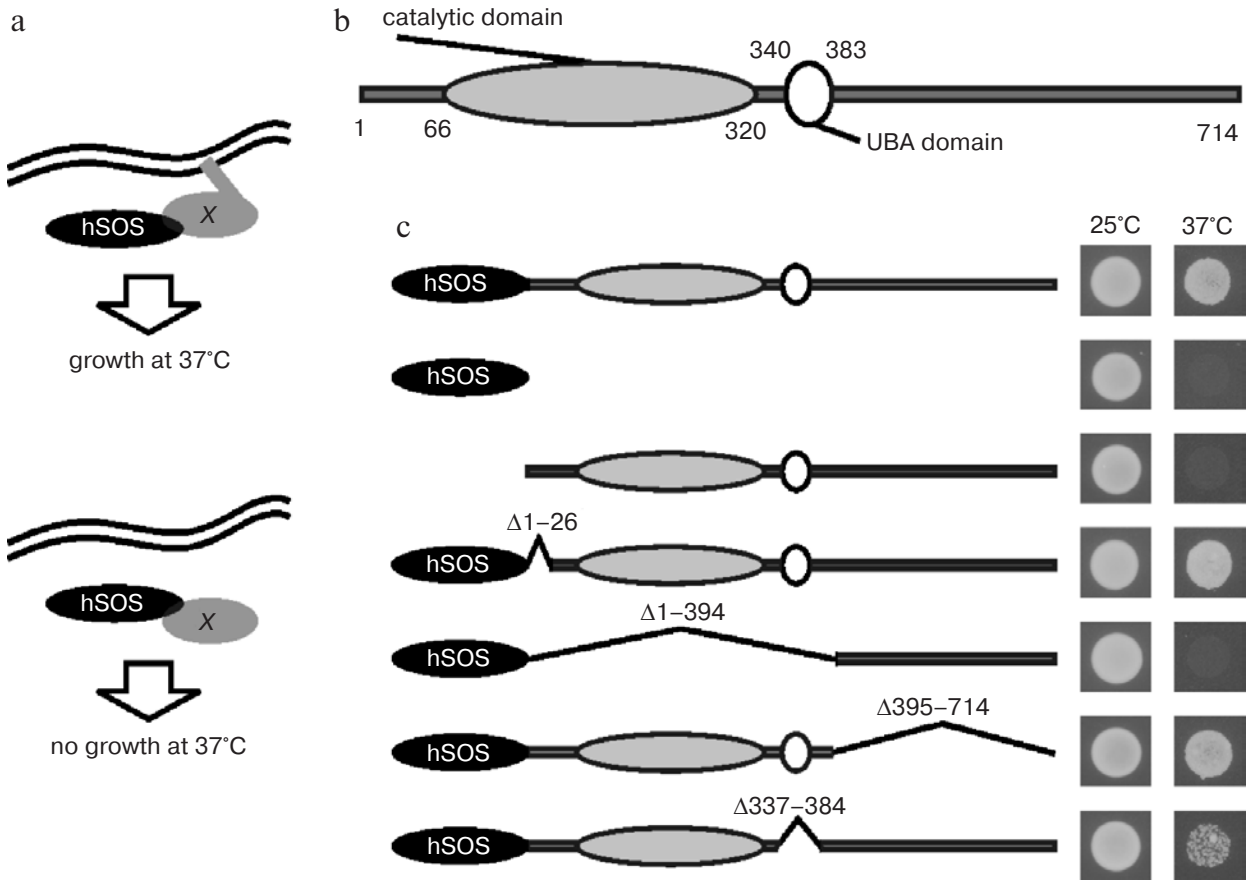

Fig. 3. Membrane localization of the MAK-V protein in yeast. a) Schematic representation of yeast-based assay for membrane localization of proteins. Membrane localization of hSOS-protein X chimera due to membrane targeting of protein X results in complementation of temperature-sensitive mutation and in the ability of cdc25H yeast to grow at the non-permissive temperature (37°C). If the chimeric protein fails to localize on membrane, the yeast can grow only at the permissive temperature (25°C). Thus, the ability of yeast expressing hSOS-protein X chimera to grow at 37°C points to membrane association of protein X. b) Domain organization of the MAK-V protein. Positions of catalytic and UBA domains are shown in amino acid residues. c) Membrane localization of the MAK-V protein kinase and its deletion mutants in yeast. On the left, proteins produced in yeast are schematically represented with deletions in MAK-V protein shown in amino acid residues. On the right, results of complementation of the temperature-sensitive mutation in yeast producing respective chimeric proteins are shown. Yeast were grown for two days at permissive (25°C) or for four days at non-permissive (37°C) temperature.

DISCUSSION

Targeting of protein kinases to specific intracellular compartments might be an important event for their activation and correct localization of protein kinase activity. In particular, membrane localization of a related to MAK-V protein kinase Par-1 is essential for its function as a regulator of cell polarity and is under control in the cell [16, 17]. In this work, we demonstrated that a significant part of MAK-V protein kinase cellular pool is associated with membranes in mammalian cells. Together with results of analysis in cdc25H yeast, these data allowed us to conclude that MAK-V can localize to cellular membranes. As only a portion of intracellular MAK-V pool is associated with membranes, we can expect that this association is not constitutive—rather it is subjected to regulation. The importance and significance of the revealed association of MAK-V with membranes for functions of this protein kinase and its catalytic activity are yet to be investigated, as well as the molecular mechanisms underlying membrane targeting of MAK-V.

The ability of MAK-V to localize on membrane should rely on specific determinants in the polypeptide chain that mediate interaction with membrane. Amino acid sequence analysis did not reveal any sites of covalent modifications leading to membrane association of proteins such as myristylation (including internal glycine residues), palmitoylation, and prenylation, thus suggesting that MAK-V localization on membranes is mediated by noncovalent interactions with membrane components. At the same time, observed in yeast membrane targeting of vertebrate MAK-V protein suggests that these membrane components are highly evolutionarily conserved. These partners for interaction with MAK-V could be conserved membrane-associated proteins or non-protein moieties. The latter are represented by phosphatidylinositol phos-

phates, which serve as a membrane interface for interaction with various proteins [18-23]. Primary structure analysis showed that MAK-V lacks any conventional phosphatidylinositol-binding domain. However, the catalytic domain of MAK-V contains a stretch of positively charged amino acids ^{91}KVIDKKRAKK which fits a phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P_2) consensus binding site [24, 25]. Although catalytic lysine-91 is one of the invariant residues within protein kinase catalytic domain, the remaining part of this sequence enriched in positively charged residues is unique for MAK-V and is not found in other AMPK-related protein kinases whose membrane localization was not reported. Taking into account that this lysine/arginine sequence is conserved in human, mouse, frog, and fish MAK-V proteins, it could be suggested that this is a determinant mediating interaction between MAK-V and PI(4,5)P_2, thus being responsible for association of the protein kinase with membrane. However, this suggestion is yet to be proved experimentally.

Our experiments demonstrated the requirement for UBA domain for efficient association of MAK-V with membranes, thus pointing to an important role of UBA domain in regulation of MAK-V protein kinase intracellular localization. Interestingly, previous studies revealed that UBA domain is required for nuclear targeting of another AMPK-related protein kinase, SIK [26]. Results of our study further exemplify the role played by UBA domains of AMPK-related protein kinases in determining their association with specific intracellular compartments.

The observed dependence of MAK-V membrane association on the presence of UBA domain is not controversial to our hypothesis that membrane targeting is mediated by ^{91}KVIDKKRAKK sequence in the catalytic domain. UBA domains of AMPK-related protein kinases possess unique for UBA domain function. Unlike UBA domains in other proteins, they do not bind ubiquitin but rather play an important conformational role and are required for activation of AMPK-related protein kinases [26, 27]. Provided that presence of UBA domain is important for correct conformation of the catalytic domain, deletion of the UBA domain in MAK-V protein kinase might lead to conformational changes and/or alter accessibility of the region containing the sequence ^{91}KVIDKKRAKK, thus influencing the ability of MAK-V to interact with PI(4,5)P_2 and localize to membrane.

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