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Interaction between MAK-V Protein Kinase and Synaptopodin

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Abstract—MAK-V protein kinase (also known as HUNK) was discovered more than decade ago but its functions and molecular mechanisms of action still remain mostly unknown. In an attempt to associate MAK-V with particular chains of molecular events, we searched for proteins interacting with the C-terminal domain of MAK-V protein kinase. We identified synaptopodin as a protein interaction partner for MAK-V and confirmed this interaction in various ways. Because synaptopodin is important for dendritic spine formation and plays a role in synaptic plasticity, our results might have significant impact on future studies for understanding the role of MAK-V in cells of the nervous system.

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Key words: MAK-V/HUNK protein kinase, synaptopodin, protein–protein interactions, brain

MAK-V protein kinase belongs to a group of AMPK-like protein kinases. MAK-V was identified [1] and cloned [2, 3] more than a decade ago but its molecular mechanisms of action and participation in particular molecular processes remain mostly unknown. One of the effective ways to associate a protein with particular molecular processes is to search for its interaction partners. Earlier we have applied this approach for MAK-V using protein interaction cloning in yeast [2, 4, 5]. A number of proteins that can be potential interaction partners for MAK-V were identified, and the involvement of MAK-V in endosomal transport regulation was demonstrated based on detection of interaction between MAK-V and rabaptin-5 protein [2]. It is known that regions of protein kinases beyond the catalytic domain often play a regulatory role and serve as an interface for intramolecular interactions or interactions with other proteins that can determine their integration into specific cascades of molecular events. This makes it especially attractive to search for proteins interacting with the unique C-terminal domain of MAK-V (Fig. 1). In this work we searched for proteins interacting with the C-terminal domain of the MAK-V protein kinase. We used a proteomic approach which is based on affinity purification of proteins on immobilized recombinant C-terminal domain of MAK-V followed by their identification using mass-spectrometric analysis and confirmation of specificity of the revealed interactions.

Abbreviations: GAL4AD, transcription activation domain of GAL4 transcription factor; GAL4BD, DNA-binding domain of GAL4 transcription factor; GFP, green fluorescent protein; GST, glutathione S-transferase.

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MATERIALS AND METHODS

Preparation of affinity matrix with immobilized C-terminal fragment of the MAK-V protein kinase. To produce C-terminal fragment of the mouse MAK-V (amino acids 395-714), corresponding *mak-v* cDNA fragment was cloned into pGEX-4T-3 vector (GE Healthcare, Great Britain). The C-terminal fragment of MAK-V was expressed in Rozetta-gami *E. coli* (Novagen, USA) as a chimera with glutathione S-transferase (GST). The recombinant protein was purified on glutathione-Sepharose (GE Healthcare) following the manufacture's recommendations, and it was covalently linked with glutathione using disuccinimidyl suberate (Pierce, USA) [6] (affinity matrix GST-C-MAK-V). Control affinity matrix with GST protein was prepared in similar way.

Affinity purification of proteins. Three milliliters (1.8 mg of protein) of mouse brain peripheral membrane fraction [6] were preincubated with control affinity matrix with immobilized GST protein for 30 min at 4°C to sorb nonspecifically binding proteins. Then the sample was incubated with 20 μl GST-C-MAK-V or control

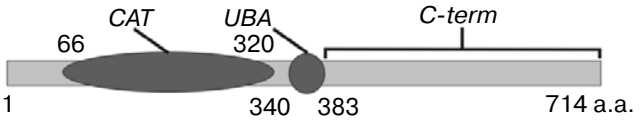

Fig. 1. Domain structure of the MAK-V protein kinase. Catalytic (CAT), ubiquitin-associated (UBA), and C-terminal (C-term) domains are shown. Domain boundaries are indicated by amino acids sequence numbers (a.a.).

affinity matrix for 1 h at 4°C. After washing with 1× Tris-buffered saline (Sigma, USA), bound proteins were eluted by boiling in SDS-PAGE sample buffer and resolved in a 7.5% polyacrylamide gel with subsequent Coomassie blue R250 staining. Proteins bands were cut from the gel, and the proteins were identified with MALDI-TOF spectrometry.

Mass-spectrometric analysis. Proteins bands cut from the polyacrylamide gel were cleaved by trypsin for their further analysis by the peptide mass fingerprint [7]. Mass-spectrometric analysis of tryptic digests was performed using an MALDI-TOF mass spectrometer Ultraflex (Bruker Daltonics, Germany). Registration of positive ions was done in reflector mode in m/z range 600-5000 Da. Proteins were identified using the Mascot search engine (Matrix Science, USA; www.matrixscience.com).

Binding assay of proteins with full-length MAK-V protein kinase. Full-length MAK-V was immobilized on anti-FLAG-M2 affinity gel (Sigma) from extracts of PC12TetOn cells with inducible expression of mouse MAK-V protein with C-terminal FLAG epitope [8]. Control matrix was prepared using extracts of the same cells without induction of MAK-V-FLAG expression. Twenty microliters of anti-FLAG-M2 affinity gel with immobilized MAK-V-FLAG protein or control matrix were incubated with 1.5 ml (0.9 mg protein) of mouse brain peripheral membrane fraction for 1 h at 4°C. After washing with 1× Tris-buffered saline containing 0.5% Triton X-100, bound proteins were eluted by boiling in SDS-PAGE sample buffer. The presence of proteins in eluates was analyzed by Western blotting.

Western blotting. Proteins were resolved in SDS-PAGE and transferred onto Hybond P membrane (GE Healthcare) by semi-dry transfer. Western blot analysis was performed using mouse monoclonal antibodies against synaptopodin (Acris Antibodies, Germany) and STOP/MAP6 protein (Transduction Laboratories, USA), rabbit polyclonal anti-FLAG (Sigma) and anti-synapsin Ia/b (Santa Cruz Biotechnology, USA) antibodies and corresponding secondary antibodies conjugated with horseradish peroxidase (GE Healthcare). Immobilon Western reagent (Millipore, USA) was used for detection.

Immunocytochemistry assay. NCI-H1299 cells were cultivated in DMEM/F12 (1 : 1) media (HyClone, USA) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were seeded onto 16-mm round cover glasses and co-transfected with plasmids to produce MAP6 or human synaptopodin proteins as chimeras with green fluorescent protein (GFP) (OriGene Technologies, USA) and the plasmid to produce mouse MAK-V protein kinase with C-terminal FLAG epitope [9] using Unifectin-56 transfection reagent (Rusbiolink, Russia). Twenty-four hours after transfection cells were fixed in 3% neutral-buffered paraformaldehyde and stained with primary anti-FLAG antibodies and secondary antibodies against rabbit immunoglobulins conjugated with AlexaFluor546 fluorescent dye (Molecular Probes, USA) [10]. Chimeras of synaptopodin and MAP6 with GFP and MAK-V-FLAG protein stained with antibodies were visualized using a Leica DMR microscope with ×100 objective equipped with a Leica DC350F cooled CCD camera. The recorded images were processed in Photoshop 5.0 program (Adobe, USA).

Analysis of protein interaction in yeast two-hybrid system. The Matchmaker II yeast two-hybrid system (Clontech, USA) was used to analyze interaction between MAK-V and synaptopodin. Plasmid pAS-MAK-V for production of mouse MAK-V as a chimera with the DNA-binding domain of GAL4 transcription factor (GAL4BD) was generated by cloning of mouse *mak-v* cDNA fragment containing entire *mak-v* open reading frame into pAS2-1 vector (Clontech) in-frame with GAL4BD. To create pACT-SYNPO plasmid for expression of synaptopodin as a chimera with the transcription activation domain of GAL4 transcription factor (GAL4AD), human synaptopodin cDNA fragment from pCMV6-AC-GFP plasmid (clone RG214922; OriGene Technologies) coding for amino acid residues 17-903 of synaptopodin was cloned into pACT2 plasmid (Clontech) in-frame with GAL4AD (details of cloning are available on request). All manipulations with yeast were done as described in the Matchmaker II system handbook. CG1945 yeast strain was used to analyze the interaction. Interaction of proteins expressed as chimeras with GAL4BD and GAL4AD was monitored by activation of *HIS3* reporter gene which was detected based on the ability of transformants to grow in minimal media in the presence of 60 mM 3-amino-1,2,4-triazole. To monitor specificity of the reporter gene activation, growth of yeast co-transformed with pAS-MAK and pACT2 plasmids, and with pAS2-1 and pACT-SYNPO plasmids in the presence of 3-amino-1,2,4-triazole was assayed.

RESULTS

Purification and identification of proteins interacting with C-terminal domain of the MAK-V protein kinase. The glutathione-Sepharose affinity matrix with covalently immobilized recombinant C-terminal fragment of mouse

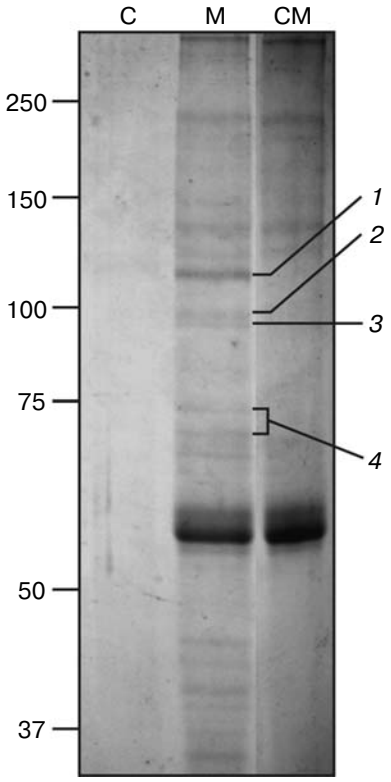

Fig. 2. Purification of proteins interacting with MAK-V C-terminal domain. Proteins from mouse brain peripheral membrane fraction purified on GST-C-MAK-V affinity matrix (M) or on control matrix (C) were resolved in 7.5% gels by SDS-PAGE with subsequent Coomassie blue R250 staining. Proteins purified only on GST-C-MAK-V affinity matrix (arrows) were identified with MALDI-TOF spectrometry: *1*) MAP6; *2*) synaptopodin; *3*) proline- and glutamine-rich splicing factor; *4*) synapsin Ia/b. Positions of molecular weight standards (in kDa) are shown on the left. CM, eluate from GST-C-MAK-V affinity matrix without incubation with peripheral membrane fraction.

MAK-V (amino acids 395-714) fused with GST protein (matrix GST-C-MAK-V) was used to purify proteins interacting with the C-terminal domain of MAK-V. Mouse brain extract was used as the source of MAK-V interacting proteins because brain is one of the organs with highest expression of MAK-V [2]. A number of proteins were repetitively purified from peripheral membrane fraction on GST-C-MAK-V affinity matrix but not on the matrix with immobilized GST protein, which was used to control specificity of purification (Fig. 2). Protein bands with mobility of about 70, 72, 74, 98, 99, and 115-kDa were cut out of the gel and identified using MALDI-TOF spectrometry. The analysis showed that the 72- and 74-kDa proteins were apparently two isoforms of synapsin I, the 98-kDa was proline- and glutamine-rich splicing factor, the 99-kDa protein was synaptopodin, and the protein with molecular weight 115 kDa was MAP6 (also known as STOP). We failed to identify the protein with molecular weight of 70 kDa.

Establishing protein authenticity and analysis of specificity of interaction with MAK-V. To establish authenticity of proteins identified with MALDI-TOF spectrometry, eluates from GST-C-MAK-V matrix were analyzed by Western blotting using antibodies specifically recognizing MAP6/STOP proteins, synaptopodin, and synapsin I. Also, the eluates from the control affinity matrix with immobilized GST protein were analyzed to monitor the specificity of interaction. The assay showed that all analyzed proteins detected with mass spectrometry actually bind to GST-C-MAK-V matrix. However, according to Western blot analysis results, it was found that MAP6/STOP protein also purifies in significant quantity on the control matrix, which suggests the absence of interaction specificity with the C-terminal domain of MAK-V protein kinase. This was confirmed by immunofluorescent analysis of transiently transfected NCI-H1299 cells which did not reveal colocalization of MAK-V with C-terminal FLAG epitope and MAP6 chimera with GFP (data not shown).

Analysis with antibodies against synapsin I showed that both synapsin I isoforms (a and b) predominantly purify on GST-C-MAK-V matrix. However, during pro-

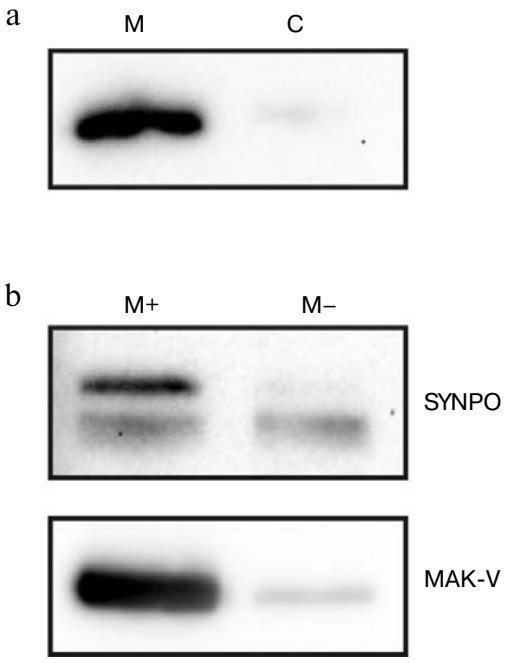

Fig. 3. Interaction of synaptopodin with MAK-V protein kinase. a) Western blot analysis of proteins from mouse brain peripheral membrane fraction purified on GST-C-MAK-V affinity matrix (M) or on control matrix (C) with antibodies against synaptopodin. b) Western blot analysis of proteins from mouse brain peripheral membrane fraction purified on anti-FLAG-M2 affinity gel with immobilized full-length MAK-V with C-terminal FLAG epitope (M+) or on control matrix (M–) with antibodies against synaptopodin (SYNPO). Below, results of Western blotting of the same membrane with anti-FLAG antibodies (MAK-V) to detect MAK-V immobilized on the matrix are shown.

tein purification from extracts of PC12 cells expressing endogenous synapsin I on anti-FLAG-M2 affinity gel with immobilized full-length MAK-V, it was established that synapsin I binds both to anti-FLAG-M2 affinity gel with immobilized MAK-V and to the control affinity gel (data not shown). This result fails to confirm specificity of the detected interaction between MAK-V protein kinase and synapsin I although it does not rule out this possibility.

Western blot analysis of proteins purified on GST-C-MAK-V and on control matrix with immobilized GST protein using antibodies against synaptopodin showed that the antibodies detected an approximately 99 kDa protein which is consistent with the experimentally observed mobility of brain synaptopodin [11]. Importantly, staining of synaptopodin was observed only in eluate from matrix with immobilized MAK-V C-terminal domain (Fig. 3a). Therefore, the results indicate that synaptopodin specifically interacts with C-terminal domain of the MAK-V kinase.

Interaction of synaptopodin with full-length MAK-V protein kinase. Results of the experiments showed that synaptopodin interacts with C-terminal domain of the MAK-V protein kinase. However, the C-terminal domain alone can have a conformation distinct from the conformation in full-length protein. To confirm that the detected interaction is not artificial due to this reason, the interaction of synaptopodin with full-length MAK-V was analyzed. Full-length MAK-V protein with C-terminal FLAG epitope was purified using anti-FLAG-M2 affinity gel from PC12TetOn cell clone with induced expression of MAK-V-FLAG protein [8]. Affinity gel with immobilized through FLAG epitope MAK-V protein was used to precipitate synaptopodin from mouse brain peripheral membrane fraction. Processed in a similar way anti-FLAG affinity gel but using lysates of cells in which expression of MAK-V-FLAG protein was not induced, was used as the control of interaction specificity. It was established that synaptopodin could bind to anti-FLAG-M2 affinity gel only if MAK-V was immobilized on it (Fig. 3b). Therefore, synaptopodin interacts not only with the C-terminal domain but also with the full-length MAK-V protein.

For further confirmation of interaction between MAK-V and synaptopodin, their colocalization in cells was analyzed. NCI-H1299 cells were transiently transfected with plasmids for production of MAK-V protein with C-terminal FLAG epitope, which was used to detect MAK-V, and chimera of synaptopodin with GFP. Synaptopodin detected by GFP fluorescence (Fig. 4a) was localized in the cell cytoplasm on actin filaments, which agrees with the published data [12]. MAK-V in NCI-H1299 cells localized to small vesicles distributed through the cytoplasm (Fig. 4b). However, in some regions, especially on the cell periphery where the density of synaptopodin-positive structures is not high, a pro-

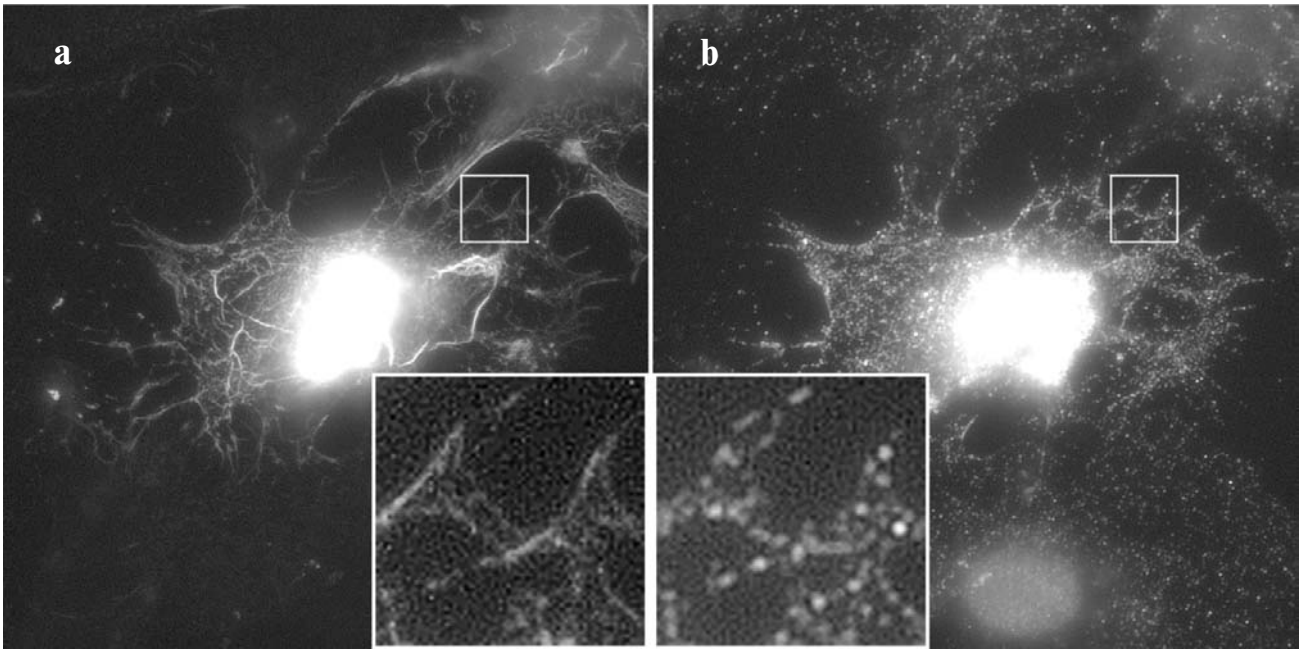

Fig. 4. Colocalization of MAK-V protein kinase and synaptopodin in cells. Immunofluorescence assay of NCI-H1299 cells transiently producing GFP-synaptopodin detected by green fluorescence of GFP (a) and MAK-V with C-terminal FLAG epitope detected with primary anti-FLAG antibodies and secondary antibodies labeled with AlexaFluor546 (b). Insets show that MAK-V-positive vesicles predominantly localized along the synaptopodin-positive fibrils.

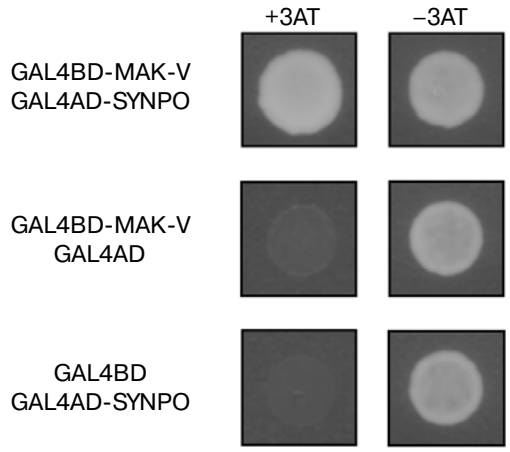

Fig. 5. Interaction of synaptopodin and MAK-V protein kinase in yeast. The results of analysis of *HIS3* reporter gene activation by the ability of yeast expressing MAK-V fused to GAL4BD or GAL4BD alone and synaptopodin fused to GAL4AD or GAL4AD alone in indicated combinations to grow in the presence (+3AT) or absence (–3AT) of 3-amino-1,2,4-triazole.

nounced tendency for localization of MAK-V-positive vesicles along synaptopodin-positive fibrils was observed (Fig. 4). Such pattern of staining suggests the possibility of a physical interaction between MAK-V and synaptopodin, and indirectly confirms the conclusion about interaction of MAK-V with synaptopodin.

Finally, another heterologous system for analysis of protein interaction, namely the yeast two-hybrid system, was used to confirm interaction between MAK-V and synaptopodin. As shown in Fig. 5, co-expression of GAL4BD fused to MAK-V and GAL4AD fused to synaptopodin in yeast resulted in activation of *HIS3* reporter gene, thus indicating that MAK-V interacts with synaptopodin. At the same time, *HIS3* activation was absent in yeast co-expressing GAL4BD fused to MAK-V and GAL4AD alone or GAL4BD alone and GAL4AD fused to synaptopodin; this suggests the absence of reporter gene transactivation and indicates specificity of the observed interaction.

DISCUSSION

In this work we searched for proteins interacting with the C-terminal domain of the MAK-V protein kinase. As a result, synaptopodin was isolated as an interaction partner of MAK-V, and the specificity of the detected interaction of these two proteins was confirmed. Synaptopodin is now the only protein for which interaction with the MAK-V C-terminal domain has been demonstrated. Domains of protein kinases beyond their catalytic domains often regulate their protein kinase activity and/or determine their correct intracellular localization. Interaction of MAK-V with synaptopodin might have one of these functions though this needs further investigation. Synaptopodin is expressed *in vivo* in brain telencephalonic neurons and in kidney podocytes [11]. Whereas synaptopodin [13] and MAK-V [14] play particular roles in kidneys, their functional link in this organ is improbable. The reason for this is that expression of synaptopodin is typical for kidney podocytes [11], and MAK-V is produced only in distal renal tubule cells [14]. In brain, synaptopodin is associated with specific structures present in dendritic spines which form synaptic contacts [11]. The functional role of synaptopodin in these structures remains unknown but genetic knockout of synaptopodin leads to loss of dendritic spines concomitant with decrease in long-term potentiation and disturbance of spatial learning thus suggesting that synaptopodin plays a role in synaptic plasticity [15]. It is interesting to note that expression of MAK-V is rather high in brain structures expressing synaptopodin, such as hippocampus, cerebellar cortex, striatum, and olfactory bulb [2, 11], though the presence of MAK-V in dendritic spines was not investigated. Results of research on MAK-V suggest that this protein kinase can play a role in the nervous system [2, 3, 16, 17]. Taking into account the role of synaptopodin in brain, the detected interaction of MAK-V with synaptopodin can be a starting point for establishing the role of MAK-V in the brain. On one side, synaptopodin can act as a platform for localization of MAK-V on dendritic spines. On the other side, the interaction between MAK-V and synaptopodin may have functional consequences for synaptopodin itself. Synaptopodin is able to interact with F-actin and α-actinin [12] regulating the actin-binding ability of the latter [13]; it also participates in regulation of the RhoA signal pathway and in migration of podocytes [18]. Synaptopodin itself is phosphorylated which makes possible its interaction with 14-3-3β protein. This interaction prevents cathepsin-L-mediated degradation of synaptopodin [19]. In this context, an especially attractive hypothesis is that MAK-V is a protein kinase that phosphorylates synaptopodin in neurons thereby regulating its stability and functions in dendritic spine formation and providing long-term potentiation of synapses.

In conclusion, we have demonstrated that MAK-V protein kinase is able to interact with synaptopodin. This result can have substantial significance for further investigation of the role of MAK-V in nervous system cells.

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