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Mover is a novel vertebrate-specific presynaptic protein with differential distribution at subsets of CNS synapses

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Abstract **Presynaptic nerve terminals contain scaffolding proteins that orchestrate neurotransmitter release at active zones. Here we describe mover, a yet unknown non-transmembrane protein that is targeted to presynaptic terminals when overexpressed in cultured neurons. Confocal immunomicroscopy revealed that mover colocalizes with presynaptic markers in the calyx of Held. In the hippocampus, mover localizes to mossy fibre terminals, but is absent from inhibitory nerve terminals. By contrast, mover localizes to inhibitory terminals throughout the cerebellar cortex. Our results suggest that mover may act in concert with generally expressed scaffolding proteins in distinct sets of presynaptic terminals.**

Keywords: Presynaptic; Hippocampus; Cerebellum; Calyx of Held

1. Introduction

Synapses of the central nervous system are specialized sites of cell–cell contact. The presynaptic site is designed to allow for highly regulated, exocytotic neurotransmitter release from synaptic vesicles (SVs) [1]. The majority of proteins involved in neurotransmitter release are evolutionarily conserved among vertebrates and invertebrates and include two types of protein complexes, which are crucially involved in neurotransmitter release: first, SNARE-complexes, built from the SV-transmembrane protein VAMP and the plasmamembrane proteins syntaxin-1 and SNAP25 which mediate exocytotic fusion of SVs with the plasmamembrane [2]. Second, the cytomatrix of active zones (CAZ) which is thought to represent a supramolecular complex serving several functions, including formation of a scaffold for the recruitment of additional proteins by organizing presynaptic protein–protein interactions, regulation of SNARE-complexes, and spatial restriction of SV fusion to active zones [3–5].

The expression of two related multidomain CAZ-proteins, namely bassoon and piccolo/aczonin, is restricted to vertebrates and therefore may add vertebrate-specific features to presynaptic nerve terminals [5]. To identify novel vertebrate specific presynaptic proteins, we performed a yeast-2-hybrid screen using one of the cognate vertebrate specific CAZ-components, namely bassoon, as bait.

Here, we describe the tissue distribution and subcellular localization of a putative bassoon binding partner, which we call mover alluding to its association with *mo*ssy fibre terminals and its exclusive expression in *ver*tebrates. We show that mover is a presynaptic molecule with differential distribution at distinct sets of excitatory and inhibitory synapses.

2. Materials and methods

2.1. Antibodies

Primary antibodies: anti-myc (9E10; Santa Cruz), anti-MAP-2, anti-synapsin, anti-synaptophysin (Sigma), anti-bassoon (Stressgen), anti-synaptotagmin 1, anti-VGAT (Synaptic Systems). Rabbit polyclonal antibodies were raised against mover fused to glutathione-*S*-transferase (GST). Mover-specific antibodies were purified using two affinity columns (Sterogene).

2.2. Yeast-2-hybrid screen

Screening was performed using the L40 yeast strain harbouring HIS3 and β-gal as reporter gene. Nucleotide sequences encoding amino acids 3263–3938 of rat bassoon were subcloned into the lexA fusion vector pHyblexZeo and used to screen an adult mouse brain cDNA library constructed in pPC86 vector containing the GAL4 activation domain vector (Invitrogen). Approximately 2×10^7 clones of a mouse cDNA library were screened. Positive clones of the initial screen were isolated, sequenced and retransformed to validate their ability to bind to the respective bassoon fragment.

2.3. Constructs

The rat mover cDNA was cloned from rat brain total RNA by RT-PCR with oligonucleotides based on the 5′- and 3′-terminal mouse sequences, and subcloned into CMV-promotor based mammalian expression vectors (Clontech). The myc-sequence was fused to the C-terminus of full-length rat mover. Further constructs were synaptophysin-GFP and PSD-95-GFP [6,7].

2.4. Cell culture

Primary cultures of rat hippocampal neurons were prepared, maintained and transfected using the calcium phosphate method on DIV 3 or 4 as described [8].

2.5. Immunocytochemistry and fluorescence imaging

Cultured neurons were fixed in 4% paraformaldehyde, processed and viewed as described [8]. Adult rats were perfused with phosphate buffered saline (PBS) containing 4% paraformaldehyde, 2% polyvinylpyrrolidone, and 0.01% glutaraldehyde. The fixed tissue was kept in

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fixative for 1 h at 4 °C and cryoprotected by incubation in PBS with increasing sucrose concentrations, frozen in embedding medium (Tissue Tek) on dry ice and sectioned at 20 μm on a cryostat. Confocal images were acquired using a Leica TSC SP confocal microscope.

2.6. Subcellular fractionation of adult rat brain

Fractionation was performed as described [9,10]. The individual fractions were designated as follows: Hom, homogenate; P1, nuclear pellet; S1, supernatant after synaptosome sedimentation; P2, crude synaptosomal pellet; P3, light membrane pellet; S3, cytosolic fraction; LP2, crude synaptic vesicle fraction; LS2, cytosolic synaptosomal fraction; SPM, synaptic plasma membranes.

2.7. Synaptotagmin uptake assay

This assay identifies functional neurotransmitter release sites by stimulation-dependent uptake of an antibody that binds to the lumenal domain of the synaptic vesicle transmembrane protein synaptotagmin-1 [11]. Hippocampal neurons transfected with a myc-tagged mover construct were incubated for 90 s with anti-synaptotagmin-1 antibody in depolarisation buffer (44 mM NaCl, 90 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES pH 7.4, 30 mM glucose) followed by a 10 min incubation with anti-synaptotagmin-1 antibody in culture medium at 37 °C. After washing and fixation, the internalized antibodies were detected using anti-mouse secondary antibodies.

2.8. Northern blotting

A premade multiple tissue Northern blot (BD) was hybridized with ^{32}P-labelled mover cDNA for 1 h at 68 °C, washed three times with 2×SSC, 0.05% SDS, followed by two washes of 1×SSC, 0.1% SDS at 50 °C. To check for equal loading, the blot was stripped and re-probed with radioactively labelled human β-actin cDNA.

3. Results

The most abundant prey in the yeast-2-hybrid screen was a cDNA encoding a 266 amino acid protein, which we propose to name mover. Murine mover cDNA is found in the NCBI database as RIKEN cDNA 1200015A19 gene encoding a hypothetical protein (GenBank accession number NP_080664). Mover homologues exist in vertebrates, but not in invertebrates. Because rat mover sequences were not deposited, we used RT-PCR to clone a rat mover cDNA, which encodes a protein with 99% amino acid sequence identity to murine mover (Fig. 1). Sequence comparison of several homologues revealed a divergent N-terminal region followed by evolutionary conserved regions. Mover has no predicted transmembrane domains or putative membrane anchor sites (Fig. 1).

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rMover  MLQLRDTVDSAG---TSPTAVLAAGEDAGAG------RQGAGTPLR---QTLWPLNVHDP 48
mMover  MLQLRDTVDSAG---TSPTAVLAAGEDAGAG------RPGAGTPLR---QTLWPLNVHDP 48
hMover  MLQLRDSVDSAG---TSPTAVLAAGEEVGAGGGPGGGRPGAGTPLR---QTLWPLSIHDP 54
zMover  MLQLNDSVITLDP--ESPGAAVLLSADCDDG-LEVSARAGASVACCSSLQTLTPFHVHNP 57
xMover  MLQLNDSDISPAATLSPEGAEVQLSPDRGAS-------GTASSNYS--VQSVRSLTVHNP 51
        ****.*:  :      .  * :  . : . .         *.       *:: .: :*:*

rMover  TRRARVKEYFVFRPGTIEQAVEEIRAVVRPVEDGEIQGVWLLTEVDHWNNEKERLVLVTD 108
mMover  TRRARVKEYFVFRPGTIEQAVEEIRAVVRPVEDGEIQGVWLLTEVDHWNNEKERLVLVTD 108
hMover  TRRARVKEYFVFRPGSIEQAVEEIRVVVRPVEDGEIQGVWLLTEVDHWNNEKERLVLVTE 114
zMover  TMRAKVKDYFVFRPGTIEQAVNDIRTVALPSEDGEVLSVWLLAEVDHWNNEKERLVLITE 117
xMover  TQQARIKEYFVFRPGNLEQAVNDIRMVVLPTEDGEIQSVWLLTEVDHWNNEKERLVLITE 111
        * :*:::*:*******.:****::** *. * ****: .****:***************:*:

rMover  QSLLICKYDFISLQCQQVVRVALSAVDTISCGEFQFPPKSLNKREGFGVRIQWDKQSRPS 168
mMover  QSLLICKYDFISLQCQQVVRVALSAVDTISCGEFQFPPKSLNKREGFGVRIQWDKQSRPS 168
hMover  QSLLICKYDFISLQCQQVVRIALNAVDTISYGEFQFPPKSLNKREGFGIRIQWDKQSRPS 174
zMover  RSLLVCKYDFINLQCQQVIRISLNAVDTISIGEFEFPPKSLNKREGTGIRVQWDKRPRAS 177
xMover  RTLLICKYDFICLECQQVIRVALNAVDTISIGEFEFPPMSLNRREGIGIRILWSTR-RFS 170
        ::**:****** *:****:*::*.****** ***:*** ***:*** *:*: *..: * *

rMover  FINRWNPWSTNMPYATFIEHPMAGMDEKTASLCHLESFKALLIQAVKKAQKESPLPGQAN 228
mMover  FINRWNPWSTNMPYATFIEHPMAGMDEKTASLCHLESFKALLIQAVKKAQKESPLPGQAN 228
hMover  FINRWNPWSTNVPYATFTEHPMAGADEKTASLCQLESFKALLIQAVKKAQKESPLPGQAN 234
zMover  FMNRWNPWSTDIPYATFTEHPMAHADEKVASLCQLENFKTQLIQAVKKAHKEYPIPGRAN 237
xMover  FINKWNPWTTNMPYATFIEHPMAHADDKVMPLCKLEDFKTQLIQAVKKAHKQNPVQGKPN 230
        *:*:****:*::***** *****  *:*. .**:**.**: ********:*: *: *:.*

rMover  NVLVLDRPLLIETYVGLMSFINNEAKLGYSMTRGKIGF 266
mMover  TVLVLERPLLIETYVGLMSFINNEAKLGYSMTRGKIGF 266
hMover  GVLILERPLLIETYVGLMSFINNEAKLGYSMTRGKIGF 272
zMover  GVLILERPLLIETYLGIMSFINNEAKLGYAMTRGKIGF 275
xMover  GVLVLERPLMIETYLGLMSLINNEAKLGYAMSRGKIGF 268
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Fig. 1. Sequence alignment of rat (r-mover) with mouse (m-mover), human (h-mover), zebrafish (z-mover) and *Xenopus laevis* (x-mover) mover. Computational analysis predicts no known functional domains or conserved motifs. Mover has a predicted phosphorylation site at aa13 (Phosphothreonine).