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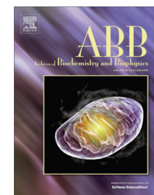

MADD/DENN/Rab3GEP functions as a guanine nucleotide exchange factor for Rab27 during granule exocytosis of rat parotid acinar cells

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ABSTRACT

We previously reported that the small GTPase Rab27 and its effectors regulate isoproterenol (IPR)-stimulated amylase release from rat parotid acinar cells. Although activation of Rab27 by a specific guanine nucleotide exchange factor (GEF) is thought to be required for amylase release, its activation mechanism is poorly understood, because GEF for Rab27 has not been reported in parotid acinar cells. In the present study, we investigated the possible involvement of MADD/DENN/Rab3GEP, which was recently described as a Rab27-GEF in melanocytes, in amylase release from rat parotid acinar cells. Reverse transcription-PCR analyses indicated that mRNA of DENND family members, including MADD, was expressed in parotid acinar cells. MADD protein was also expressed in the cytosolic fraction of parotid acinar cells. Incubation of an antibody against the C-terminal 150 amino acids of MADD (anti-MADD-C antibody) with streptolysin O-permeabilized parotid acinar cells caused not only inhibition of IPR-induced amylase release but also reduction in the amount of GTP-Rab27. Our findings indicated that MADD functions as a GEF for Rab27 in parotid acinar cells and that its GEF activity for Rab27, which is GDP/GTP cycling, is required for IPR-induced amylase release.

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Introduction

Rab proteins are believed to play important roles in intracellular membrane trafficking [1–4]. More than 60 distinct Rab isoforms have been identified in mice and humans, and these proteins comprise a large subfamily that is part of the Ras superfamily of small GTPases [5–7]. Rab activity is regulated by GDP/GTP cycling [1–4,8], with GTP-Rab and GDP-Rab representing active and inactive forms of Rab, respectively. Individual Rabs interacted with a specific effector/regulator, and these complexes regulate different steps in or types of membrane trafficking in cells, functioning as either an accelerator or a brake [9,10]. Rabs are activated by specific guanine nucleotide exchange factors (GEFs)[1] that stimulate the release of GDP and the binding of GTP. Bound GTP is then hydrolyzed by the intrinsic Rab-GTPase activity of Rabs, which is activated by GTPase-activating proteins (GAPs), the enhancers of Rab-GTPase activity. Following inactivation of Rabs, GDP-Rabs are extracted from membranes by Rab-specific GDP dissociation inhibitor (GDI), which directly binds prenylated GDP-bound Rabs and retains them in the cytosol for subsequent membrane attachment by inhibiting GDP/GTP exchange of Rabs [9,11,12].

During the past decade, a variety of putative Rab-GEFs have been identified and classified into several groups based on their structure or similarity with putative Rab-GEF domains (reviewed in [13]). The DENND [DENN (differentially expressed normal versus neoplastic)/MADD (MAP-kinase activating death domain protein) domain-containing] family constitutes the largest group of Rab-GEFs [14,15], some of which are involved in diverse signaling pathways [16]. Eighteen genes encode DENNDs in humans, and they are further classified into eight subgroups, DENND1-6, MTMR5/13 (myotubularin related protein 5 and 13), and MADD (also called DENN; hereafter simply referred to as MADD), based on their Rab-GEF specificity [14,15]. MADD was originally purified as a Rab3-GEF, Rab3 GDP/GTP exchange protein (Rab3GEP) from rat brain [17], and it was also identified as a component of a signaling protein complex that localizes to the cytosol and exerts multiple functions by binding different partners [18–20]. Recently MADD (and its *Caenorhabditis elegans* homologue AEX-3) has been shown to have GEF activity towards Rab27 in vitro and in

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[1] *Abbreviations used:* DENN, differentially expressed normal versus neoplastic; MADD, MAP-kinase activating death domain protein; DENND, DENN/MADD domain containing; GEF, guanine nucleotide exchange factor; Rab3GEP, Rab3 GDP/GTP exchange protein; GDI, GDP dissociation inhibitor; GAP, GTPase-activating protein; GFP, green fluorescent protein; IPR, isoproterenol; SLO, streptolysin O; GST, glutathione *S*-transferase; SHD, synaptotagmin-like protein homology domain; Slac2, Slp homologue lacking C2 domain; LPM, luminal plasma membrane; RT, reverse transcription; SGM, secretory granule membrane; Slp4, synaptotagmin-like protein 4; p-APMSF, 4-amidinophenylmethanesulphonyl fluoride hydrochloride; EGTA, ethylene glycol-bis (β-aminoethylether)N,N,N′,N′-tetraacetic acid.

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melanocytes [15,21,22], indicating that MADD functions as a dual GEF for both Rab3 and Rab27.

Rab27 regulates secretory granule exocytosis in a variety of secretory acinar cell types as well as melanosome transport in melanocytes (reviewed in [23]). We have previously shown that Rab27 and its effectors, synaptotagmin-like protein 4-a (Slp4-a)/granuphilin-a, Slp homologue lacking C2 domain-c (Slac2-c)/MyRIP, and Noc2 are involved in the control of isoproterenol (IPR)-induced amylase release from parotid acinar cells [24–26]. We have also identified a Tre-2/Bub2/Cdc16 (TBC) domain-containing protein, EPI64, as a GAP for Rab27 in parotid acinar cells [27]. EPI64 is mainly localized at the LPM and promotes the GTPase activity of Rab27 when acinar cells are stimulated with IPR. Inactivated GDP-Rab27 is then released from the LPM due to the function of GDI [28]. Although GDP-Rab27 in the cytosol must be re-activated by certain Rab27-GEFs to prepare for next round of amylase release, nothing is known about the function of Rab27-GEF in parotid acinar cells.

In the present study, we investigated the mRNA expression of the DENND family members, the subcellular localization of MADD protein, and the possible involvement of the Rab27-GEF function of MADD, a putative Rab27-GEF, in IPR-induced amylase release from rat parotid acinar cells.

Materials and methods

Materials

Anti-Rab27A/B and anti-MADD rabbit polyclonal antibodies were purchased from Immuno-Biological Laboratories Ltd. (Takasaki, Japan) and AnaSpec Inc. (San Jose, CA, USA), respectively. Anti-green fluorescent protein (GFP) rabbit polyclonal antibody was obtained from MBL (Nagoya, Japan). Rabbit IgG, PCR primers, and other chemical products were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rat tissue cDNA QUICK-clone™ was from TAKARA Bio Inc. (Shiga, Japan). Streptolysin O (SLO) was from Bio-Academia (Osaka, Japan).

Preparation of total RNA and subcellular fractionation of parotid acinar cells

All animal protocols were devised and performed in accordance with the Guidelines of the Nippon Dental University for the Care and Use of Laboratory Animals. Parotid acinar cells were prepared from parotid glands of male Wistar rats (approximately 10 weeks old) with enzyme digestion using trypsin (Sigma–Aldrich) and collagenase (CLSPA; Worthington Biochemical Co., Lakewood, NJ, USA) as described [29]. Total RNA from parotid acinar cells was prepared with an RNeasy® Plus Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Subcellular fractions were prepared from a homogenate of parotid acinar cells as described [28]. Specifically, parotid acinar cells were homogenized in a 20-fold volume of buffer A (5 mM HEPES–NaOH buffer (pH 7.2) containing 50 mM mannitol, 0.25 mM $MgCl_2$, 25 mM β-mercaptoethanol, 0.1 mM ethylene glycol-bis (β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA), 2 μM leupeptin, 2.5 μg/ml trypsin inhibitor, 0.1 mM 4-amidinophenylmethanesulphonyl fluoride hydrochloride (p-APMSF), 5 mM benzamidine, and 2 μg/ml aprotinin) using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 9750*g* for 10 min at 4 °C. The supernatant was then re-centrifuged at 35,000*g* for 30 min at 4 °C, and then further centrifuged at 100,000*g* for 1 h. The supernatant was considered the cytosolic fraction. The pellet from the 35,000*g* centrifugation step was suspended in buffer A containing 10 mM $MgCl_2$ and incubated on ice for 30 min. The suspension was centrifuged at 3000*g* for 15 min, and the resulting supernatant was re-centrifuged at 100,000*g* for 1 h. The resulting precipitate was considered the luminal plasma membrane (LPM) fraction, which was characterized by a specific enzyme activity, γ-glutamyl transpeptidase activity [30,31]. A secretory granule membrane (SGM) fraction was prepared by centrifugation in 40% Percoll gradient [32]. Protein assays were performed using a protein assay kit (Bio-Rad, Hercules, CA, USA).

Reverse transcription (RT)-PCR

Parotid cDNA, which was prepared from total RNA of rat parotid acinar cells, QUICK-clone™ (TAKARA Bio Inc. Kyoto, Japan) of rat brain and kidney were used as PCR templates. RT of total RNA from parotid acinar cells was performed using a Transcriptor® First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). Synthesized cDNA was made from 1 μg total RNA, and 1/20th of the RT reaction mix was used for PCR. PCR was performed using KOD-plus DNA polymerase (TOYOBO, Osaka, Japan) and specific primers for the *Dennd* family and control *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) (Table 1) under the following conditions: 30 cycles of denaturation at 94 °C for 20 s; annealing at 60 °C for 20 s; and extension at 72 °C for 30 s.

Plasmid construction

cDNAs encoding human MADD/DENN/KIAA0358, DENND2A/KIAA1277, DENND4B/KIAA0476, and DENND5A/KIAA1091 were obtained from Kazusa DNA Research Institute (Chiba, Japan). The mouse DENND1A cDNA and DENND3 cDNA were prepared as described [33,34]. The open reading frame of these cDNAs was amplified by PCR and subcloned into the pEGFP-C1 vector (TAKARA Bio Inc.) using conventional molecular biology techniques. Truncated mutants of MADD, MADD-N (N-terminal 165 amino acids), and MADD-C (C-terminal 150 amino acids) were similarly prepared with PCR, and the cDNA fragments obtained were subcloned into the pGEX-4T-3 vector (GE Healthcare, Little Chalfont, UK). Truncated mutants of DENND1A (DENND1A-N; N-terminal 1–91 amino

Table 1
Primers for PCR amplification of DENND family members.

DENND synonym (Gene)	Databank accession no.	Forward primer	Reverse primer	Product size (bp)
DENN/MADD/Rab3GEP (*Madd*)	NM_053585	CCTCAAGTGCACAGTCCTCA	TTAGCTGAGCCATTGCCTTT	218
DENND1A (*Dennd1a*)	NM_001191747	GCATGTGTACATCCCTGTGC	CCTGTGGTTGTGGAGACCTT	243
DENND1B (*Dennd1b*)	XM_222678	CAACCATCCCGGAGAGTAGA	TGTTGCCAGTACATCGGGTA	184
ST5 (*St5: Dennd2b*)	NM_001107547	CACTGAGCAGAGTGGGATGA	TTTGGAGGTGGGGTCTGTAG	184
DENND2C (*Dennd2c*)	NM_001191569	CTCCAGGATCTTCCCATTGA	TCACATCTTGCGAAAACTGC	178
DENND2D (*Dennd2d*)	NM_001107714	ACCACGGAAACTTCAGGATG	ACAGAAGGAGCGTTCTTGGA	193
DENND3 (*Dennd3*)	XM_235398	GCCAGCACAGACTTGAATGA	TGCTGCTCTCGTCTTGAGAA	180
DENND4B (*Dennd4b*)	XM_342287	GTTCCAAGTGCTTGACACGA	GAGGAGTGCTCTCACCCTTG	176
DENND5A (*Dennd5a*)	NM_001107546	AGCCTCTGTGACCTTCTGGA	GCGTCAGACTTTCTCCCGTTC	176
Control (*Gapdh*)	NM_017008	AACATCATCCCTGCATCCAC	GACAACCTGGTCCTCAGTGT	233

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