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Monoclonal Antibody DL11C8 Identifies ADAM23 as a Component of Lipid Raft Microdomains

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Abstract—A disintegrin and metalloprotease protein 23 (ADAM23) is a transmembrane type I glycoprotein involved with the development and maintenance of the nervous system, including neurite outgrowth, neuronal adhesion and differentiation and regulation of synaptic transmission. In addition, ADAM23 seems to participate in immune response and tumor establishment through interaction with different members of integrin receptors. Here, we describe a novel monoclonal antibody (DL11C8) that specifically recognizes the cysteine-rich domain of both pre-protein (100 kDa) and mature (70 kDa) forms of ADAM23 from different species, including human, rodents and avian orthologs. Using this antibody, we detected both forms of ADAM23 on the cell surface of three neuronal cell lineages (Neuro-2a, SH-SY5Y and CHLA-20), with a higher relative content of ADAM23$^{100\ kDa}$. Furthermore, we demonstrate for the first time that a catalytically inactive member of the ADAM family is present in the membrane signaling platforms, namely lipid rafts. Indeed, the mature ADAM23$^{70\ kDa}$ partitions between raft and non-raft membrane domains, while the pro-protein ADAM23$^{100\ kDa}$ is mainly expressed in non-raft domains. These membranous distributions were observed in both different brain regions homogenates and primary cultured neurons lysates from mouse cortex and cerebellum. Taken together, these findings point out ADAM23 as a lipid raft molecular component.

Keywords: ADAM23, lipid rafts, disintegrins, monoclonal antibody, prion protein, integrin.

INTRODUCTION

A disintegrin and metalloprotease (ADAM) is a family of type I transmembrane glycoproteins containing a metalloprotease domain followed by disintegrin, cysteine-rich region, EGF-like, transmembrane domain and cytoplasmic tail (Giebeler and Zigrino, 2016; Seals and Courtneidge, 2003). ADAMs are involved in various biological processes, such as sperm–egg interactions, cell differentiation, cell migration, axonal growth, muscular development, and some aspects of immunity. Besides physiological roles, members of this family are involved in pathological conditions, such as cancer, neurological and cardiac diseases, asthma, infection, and inflammation (Edwards et al., 2008; Murphy, 2008; Giebeler and Zigrino, 2016; Seals and Courtneidge, 2003).

ADAM23 (MDC3) is an ADAM family member widely expressed in the nervous system, particularly in neurons and Schwann cells of the peripheral nervous system (Dhaunchak et al., 2010; Goldsmith et al., 2004; Kegel et al., 2014; Markus et al., 2011; Owuor et al., 2009; Lin et al., 2008; Sagane et al., 1998; 1999). Similarly to other ADAMs, ADAM23 presents two domains important for its functions: the metalloprotease and disintegrin domains, however the metalloprotease domain is believed to be catalytically inactive since it lacks essential residues responsible for the Zn^{2+} coordination to the catalytic motif (Sagane et al., 1998). Therefore, ADAM23 is predicted to act mainly through its disintegrin domain. In fact, this domain interacts *in vitro* with αvβ3 and α4 integrin receptors, promoting neural (Cal et al., 2000) and immune (Wang et al., 2017) cell adhesion, respectively. In addition, this interaction has been shown to negatively modulate the αvβ3 activation in MDA-MB-435 cells and the loss of ADAM23 expression enhances αvβ3 integrin-mediated cell migration and adhesion (Verbisck et al., 2009).

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Abbreviations: ADAM23, a disintegrin and metalloprotease protein 23; CNS, central nervous system; conA, concanavalin A-Sepharose; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; PrPc, cellular prion protein; TNF, tumor necrosis factor; WGA, wheat germ agglutinin.

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Accordingly a pathological role for ADAM23 in tumor progression is now emerging, since *adam23* gene is silenced in gastric, breast, brain and lung tumors by promoter hypermethylation (Costa et al., 2003; Costa et al., 2005; Takada et al., 2005; Hu et al., 2011; Verbisck et al., 2009).

ADAM23 interacts with LGI1 and LGI4 (leucine-rich glioma-inactivated proteins), two proteins that play important roles in the nervous system, as mutations in these genes have been associated with epilepsy and myelination (Sagane et al., 2008; Owuor et al., 2009). Interestingly, the interaction between ADAM23 and LGI1 stimulates neurite outgrowth and dendritic arborization (Owuor et al., 2009). However, while LGI1 involvement in myelination has been established, ADAM23 does not seem to be required for peripheral nervous system myelination (Kegel et al., 2014). Also, *adam23* was identified as a possible candidate gene on the risk to canine epilepsy, however the described single variant ADAM23-R387H seems to be a polymorphism rather than a causative mutation in idiopathic epilepsy in dogs (Sepälä et al., 2012). Furthermore, ADAM23 knockout mice develop severe tremors and ataxia and do not survive past postnatal day 15, suggesting that this protein is also important in the development and maintenance of the nervous system (Mitchell et al., 2001; Owuor et al., 2009; Kegel et al., 2014). However, ADAM23 distribution in the cell membrane remains still elusive. Because we previously described the interaction between cellular prion protein (PrP^c) and ADAM23 (Costa et al., 2009) and PrP^c is a lipid rafts resident protein (Keshet et al., 2000; Taylor and Hooper, 2006; Watt et al., 2014), we reasoned that ADAM23 is also present in these membranous signaling platforms. To this end, we produced a novel monoclonal antibody (DL11C8) that specifically recognizes the cysteine-rich domain of ADAM23. With this novel tool, here we show that ADAM23 transits between raft and non-raft membrane domains in both homogenates of different dissected brain regions and primary cultured neurons from mouse cortex and cerebellum, indicating that ADAM23 membrane partitioning could be involved in signal transduction at the neuronal cell surface. Taken together, these findings point out ADAM23 as a new neuronal lipid raft component.

EXPERIMENTAL PROCEDURES

Cell culture

P3-X63-Ag8.653 myeloma cell line was maintained using RPMI 1640 (Gibco) supplemented with 20% fetal bovine serum (Gibco). HEK 293T and neuroblastoma cells lines (Neuro-2a, SH-SY5Y and CHLA-20) were cultured using DMEM (Gibco) with 10% fetal calf serum (FCS). MDA-MB 435 cell line was cultured using RPMI 1640 with 10% FCS. For MDA-MB 435 ADAM23 knockdown cell (435-1C, Verbisck et al., 2009) the medium was supplemented with 1 mg/mL G418 (Gibco). MCF-7 cell line was maintained using MEM (Sigma) with 10% FCS. The cells were grown at 37 °C under 5% CO_2. CHLA-20 cell line was a generous gift from Dr. Selene E. Esposito (Pontificia Universidade Catolica do Paraná – Brazil). HEK 293T cells were transfected with expression vectors by calcium phosphate co-precipitation method (Costa et al., 2009). The expression vectors (pCMV6-Entry C-terminal Myc-FLAG tags) for mouse ADAM11 (NM_009613), ADAM22 (NM_001007220) and ADAM23 (NM_001177600) were purchased from OriGene Technologies, Inc. (MD, USA). Human ADAM23 was expressed with pcDNA-*ADAM23*-HA (Cal et al., 2000).

Cloning, expression and purification of recombinant proteins

A 648 bp fragment of human *ADAM23* (NM_003812.3) cDNA corresponding to the disintegrin and cysteine-rich domain was amplified from pcDNA-*ADAM23*-HA (Cal et al., 2000) by PCR using the following primers: forward 5′-CGGGATCCGAAGCTGGGGAGGAGTGTG-3′ and reverse 5′-CCCAAGCTTTCATAGGCACTTCCGATC TAA-3′. PCR (25 μL) was carried out with 1 U Pfu polymerase, 2 mM $MgS0_4$, 0.5 μM of each primers and 1 ng template. The conditions of thermal cycling were 35 cycles of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 3 min. The fragment amplified was digested with *BamHI* and *HindIII* and cloned into pET28a vector (Novagen). Human recombinant $_6$His-ADAM23$_{511–726}$ was expressed in BL21 Star (DE3) *Escherichia coli* for 3 h at 37 °C using 1 mM isopropyl β-D-thiogalactoside (IPTG). The recombinant protein expressed in bacterial cells was purified by immobilized-metal affinity chromatography under denaturing conditions using Ni-NTA agarose column according to the manufacturer's instructions (Qiagen). Purified recombinant protein was extensively dialyzed against PBS and identified by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting (WB) using anti-penta His tag monoclonal antibody (Qiagen).

Monoclonal antibody production

The monoclonal antibodies were produced in accordance to the Committee for Animal Experimentation in the Setor de Ciências Biológicas—Universidade Federal do Paraná (protocol approval #588). Female SWISS mice were immunized 4 times with 2-week intervals by intraperitoneal injection of 40 μg of the antigen, 1 mg of aluminum hydroxide, 50 μL of PBS and 70 μL of Freund's complete adjuvant for the first immunization and incomplete for the others. The antiserum titers were determined by enzyme-linked immunosorbent assay (ELISA). After hyperimmune serum analysis, the positively immunized mice were intravenously boosted with 100 μg of antigen 3 days before hybridoma fusion procedure. Spleen lymphocytes were fused with myeloma P3-X63-Ag8.653 cells, in a 1 to 5 ratio (Yokoyama et al., 2013). These cells were then grown in HAT-supplemented medium, and culture supernatants were screened by ELISA. The secreting cells were cloned by limiting dilutions, and supernatants from stable hybridomas were analyzed by ELISA, WB blot and immunofluorescence assays. MoAb DL11C8 isotype (IgG1 kappa) was determined with the SBA Clonotyping TM System/HRP test kit (Southern Biotech, USA) according to manufactory instructions.

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