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Membrane palmitoylated proteins regulate trafficking and processing of nectins☆

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ABSTRACT

Nectins are cell–cell adhesion molecules involved in the formation of various intercellular junctions and the establishment of apical-basal polarity at cell–cell adhesion sites. To have a better understanding of the roles of nectins in the formation of cell–cell junctions, we searched for new cytoplasmic binding partners for nectin. We report that nectin-1α associates with membrane palmitoylated protein 3 (MPP3), one of the human homologues of a Drosophila tumor suppressor gene, *Disc large*. Two major forms of MPP3 at 66 and 98 kDa were detected, in conjunction with nectin-1α, suggesting that an association between the two may occur in various cell types. Nectin-1α recruits MPP3 to cell–cell contact sites, mediated by a PDZ-binding motif at the carboxyl terminus of nectin-1α. Association with MPP3 increases cell surface expression of nectin-1α and enhances nectin-1α ectodomain shedding, indicating that MPP3 regulates trafficking and processing of nectin-1α. Further study showed that MPP3 interacts with nectin-3α, but not with nectin-2α, showing that the association of nectins with MPP3 is isoform-specific. MPP5, another MPP family member, interacts with nectins with varying affinity and facilitates surface expression of nectin-1α, nectin-2α, and nectin-3α. These data suggest that wide interactions between nectins and MPP family members may occur in various cell–cell junctions and that these associations may regulate trafficking and processing of nectins.

Introduction

Formation of cell–cell junctions, such as tight junctions in epithelial cells or puncta adherens junctions at synapses, requires the assembly of a highly ordered protein complex containing receptors, signaling molecules, and scaffolding proteins. These assemblies play roles in regulating various cellular processes that govern cellular functions and tissue organization. One of the major key players for the assembly and maintenance of cell–cell junctions is cell adhesion molecules (CAMs) (Dalva et al., 2007; McAllister, 2007; Niessen, 2007).

Recently, nectin, a member of immunoglobulin (Ig)-like CAMs, has been described as a major organizer for the various cell junctions (Takai et al., 2008a; Takai and Nakanishi, 2003). The nectin family is composed of 4 members, nectin-1, -2, -3 and -4, and two or three splicing variants have been found for each isoform (Takai et al., 2003; Takai and Nakanishi, 2003). Nectins have one extracellular region composed of three Ig-like loops, one transmembrane domain, and one cytoplasmic tail (Lopez et al., 1995). Nectin-1, nectin-2 and nectin-3 are expressed ubiquitously in various cell types including epithelia, fibroblasts and neurons, whereas nectin-4 is mainly expressed in the human placenta (Takai and Nakanishi, 2003). Nectins connect to the actin-based cytoskeleton through interactions with afadin, which is an F-actin binding protein (Mandai et al., 1997; Takai and Nakanishi, 2003). Most of the nectin family has a class type II PDZ binding motif. Through this motif, nectins interact with the PDZ domain of afadin (Mandai et al., 1997; Miyahara et al., 2000; Reymond et al., 2001; Satoh-Horikawa et al., 2000). The nectin–afadin system initiates adherens junction formation in epithelial and fibroblast cells before E- or N-cadherin forms cell–cell adhesions (Hoshino et al., 2005; Sato et al., 2006). Nectins further promote the formation of tight junctions by recruiting other molecules such as JAM-A, claudin, and occludin (Fukuhara et al., 2002; Kuramitsu et al., 2008). Nectin-1 and nectin-3 also interact with Par3 and localize together at adherens junctions of the neuroepithelial cells of the embryonic telencephalon (Takekuni et al., 2003). This recruitment establishes the polarity of epithelial cells.

To identify the molecular components of nectin-based cell–cell junctions, we searched for cytoplasmic proteins that interact with the intracellular domain of nectin-1α using a yeast two-hybrid system. We identified MPP3 as a new nectin cytoplasmic interacting molecule. MPP3 belongs to the membrane palmitoylated protein family that is composed of seven members (MPP1 to MPP7) (Gosens et al., 2008). MPP family proteins belong to the p55 Stardust fam-

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ily of membrane-associated guanylate kinase (MAGUK) family of proteins. The Drosophila Stardust protein is required for the establishment of cell polarity in the developing Drosophila embryos (Knust et al., 1993; Tepass and Knust, 1993). The MPP3 gene was identified by virtue of its genomic location to human chromosome 17q12-21 adjacent to the BRCA1 tumor suppressor locus (Fukuhara et al., 2003; Sakurai-Yageta et al., 2009). MPP3 localizes to the retinal outer limiting membrane by binding directly to family member MPP5 also known as Protein Associated with Lin Seven 1 (PALS1) (Kantardzhieva et al., 2006). This suggests that MPP3 may play a role in the maintenance of retinal integrity by regulation of cell adhesion between photoreceptors and Müller glial cells. MPP3 increases cell surface expression of the 5-HT_{2C} receptor and prevents its desensitization in cortical neurons (Gavarini et al., 2006). However, the majority of the biological function of MPP3 is still unknown.

In this study, we characterized the interaction between nectin-1α and MPP3 *in vivo* and *in vitro*. Nectin-1α recruits MPP3 to cell–cell contact sites and its association with MPP3 significantly increases cell surface expression and ectodomain shedding of nectin-1α, indicating that MPP3 is involved in trafficking and processing of nectin-1α. Then, we further demonstrated that MPP5, another member of the MPP family, also associates with nectins. This association recruits MPP5 to cell–cell contact sites and increases cell surface expression of nectins. These data suggest that wide interactions between nectins and MPP family members may occur in various cell–cell junctions and that these associations regulate trafficking and processing of nectins.

Materials and methods

Cell lines, antibodies, and plasmids

COS-7 and HEK 293A cells were purchased from American Type Culture Collection. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics (Invitrogen, Carlsbad, CA). For biochemical experiments, COS-7 and HEK-293 cells were plated to a final confluence of 80%. For immunofluorescence experiments, COS-7 cells were plated to a final confluence of 40%. Rabbit anti-nectin-1 antibodies were prepared as described (Lim et al., 2008). Rabbit polyclonal anti-MPP3 antibodies were produced against two synthetic peptides corresponding to the amino acid residue 90–104 (ELLQLLSTPHLRAVL) and 542–556 (DQHYGHLIDTVLVRQ) of human MPP3. Rabbit polyclonal anti-nectin-3 was produced against the amino acid residue 534–547 (VSHVDGSVISRREW) of mouse nectin-3α. Both polyclonal and monoclonal flag antibodies were purchased from Sigma. Nectin-2 antibody was purchased from the Proteintech group. Monoclonal and polyclonal v5 antibodies were purchased from AbD Serotec. PSD-95 antibody was from NeuroMab. Human MPP3, MPP5 and Nectin-2α cDNAs were purchased from Open Biosystems. PSD-95 cDNA was obtained from Dr. Morgan Sheng (Genentech, San Francisco, CA). Nectin-3α was cloned from the mouse brain cDNA library. Human nectin-1α (a gift from Dr. Patricia G. Spear, Northwestern University), nectin-2α and nectin-3α were v5 epitope-tagged at the N-terminus and inserted into the pVAX vector (Invitrogen, Carlsbad, CA). The cytoplasmic deletion mutants of nectin-1α and nectin-3α, and MPP3-ΔPDZ were generated by PCR and the sequence fidelity was confirmed by DNA sequencing.

Yeast two-hybrid screening

The Cytotrap yeast two-hybrid system was purchased from Stratagene and used according to the manufacturer's instructions. Briefly, we constructed a bait plasmid by inserting cDNA encoding cytoplasmic regions of nectin-1α (a.a. 400–522) into the pSos vector containing the human Sos gene. cDNAs encoding cytoplasmic regions of nectin-2α (a.a. 392–479) and nectin-3α (a.a. 429–549) were also inserted into the pSos vector. *Cdc25H* cells were cotransfected with the pooled bait plasmids and the mouse brain library. The expression of library cDNAs was controlled by a galactose-inducible promoter. The transformants were grown on selectable minimal glucose plates for 4–6 days at 25 °C. Subsequently, colonies were replica plated onto minimal galactose plates and incubated at 37 °C for 5–7 days. Positive colonies exhibiting efficient growth on galactose plates at 37 °C were isolated and re-tested for galactose-dependent growth at 37 °C. Library plasmids were recovered and analyzed by DNA sequencing. The specificity of interaction was tested by retransformation of *Cdc25H* cells.

Immunoprecipitation

A crude synaptosomal membrane fraction was prepared from freshly dissected adult rat brains as previously described (Trimmer, 1991). A crude rat brain synaptosomal membrane was solubilized in 1 ml of ice-cold lysis buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 1 mM iodoacetamide, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml benzamidine, and 1 mM PMSF) containing 0.2% Triton X-100 (vol/vol). The soluble fraction was separated from insoluble fractions by centrifugation. The soluble fraction was incubated with primary antibody overnight at 4 °C, then, 50 μl of 50% slurry of protein A-agarose (Santa Cruz Biotech) was added and rotated for 1 h at 4 °C. Protein A complexes were washed four times in lysis buffer and then resuspended in reducing SDS sample buffer. To perform *in vitro* immunoprecipitation, COS-7 cells were cotransfected with the relevant constructs. Forty-eight hours after transfection, cells were collected in PBS and lysed in lysis buffer (10 mM Tris buffer, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1 mM iodoacetamide, 0.2% Triton X-100) containing 1 mM PMSF and protease inhibitor (Sigma). Primary antibody against the relevant tag was added to the cell lysate and was incubated at 4 °C overnight. Protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was used to capture the antibody–protein complex.

Transient transfection of cells

Cells were transfected using LipoD293™ (SignaGen, Ijamsville, MD) according to the manufacturer's protocol. After 1 h incubation at 37 °C, the media was changed with fresh DMEM. Cells were lysed in reducing sample buffer for Western blot analysis 48 h after transfection. For the shedding experiment, 24 h after plating, COS-7 cells were cotransfected with 0.4 μg of nectin-1 and various amounts of MPP3 (0, 0.4, 0.8, 1.2, 1.6 μg, respectively). The total amount of cDNAs for each sample was 2 μg by adding the proper amount of empty pVAX vector. After 1 h incubation at 37 °C, the media was changed with fresh DMEM.

Biotin labeling of cell surface proteins

COS-7 cells were transiently transfected with nectin-1α and MPP3 and cultured for 24 h in Dulbecco's modified Eagle's medium containing 10% FBS. Cells were washed twice with PBS, and surface proteins were labeled with Sulfo-NHS-SS-Biotin (Pierce) in PBS under gentle shaking at 4 °C for 30 min. Fifty μl of quenching solution was added to cells at 4 °C and washed twice with TBS. Cells were collected in 1000 μl of lysis buffer, disrupted by sonication on ice, incubated for 5 min on ice, and clarified by centrifugation (10,000 × *g*, 2 min). To isolate biotin-labeled proteins, lysate was added to immobilized NeutrAvidin™ gel (50 μl) and incubated for 1 h at room temperature. Gels were washed three times with wash buffer and incubated for 10 min with SDS–PAGE