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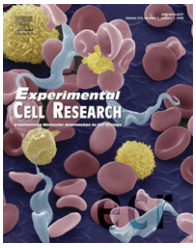

Research Article

Sorting of the v-SNARE VAMP7 in *Dictyostelium discoideum*: A role for more than one Adaptor Protein (AP) complex

Nelly Bennett[a,*], *François Letourneur*[b], *Michel Ragno*[a], *Mathilde Louwagie*[c]

[a]*Commissariat à l'Energie Atomique, iRTSV, Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, CNRS UMR5092, Université Joseph Fourier, 38054 Grenoble, France*

[b]*Institut de Biologie et Chimie des Protéines, UMR5086-CNRS/Université Lyon I, IFR 128 BioSciences Lyon-Gerland, 69367 Lyon, France*

[c]*Commissariat à l'Energie Atomique, iRTSV, Laboratoire d'Etude de la Dynamique des Protéomes, INSERM U880, Université Joseph Fourier, 38054 Grenoble, France*

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ABSTRACT

Soluble *N*-ethylmaleimide-sensitive-factor Attachment protein Receptors (SNAREs) participate in the specificity of membrane fusions in the cell. The mechanisms of specific SNARE sorting are still however poorly documented. We investigated the possible role of Adaptor Protein (AP) complexes in sorting of the *Dictyostelium discoideum* v-SNARE VAMP7. In live cells, GFP-VAMP7 is observed in the membrane of endocytic compartments. It is also observed in the plasma membrane of a small proportion of the cells. Mutation of a potential dileucine motif dramatically increases the proportion of cells with GFP-VAMP7 in their plasma membrane, strongly supporting the participation of an AP complex in VAMP7 sorting to the endocytic pathway. A partial increase occurs in knockout cells for the medium subunits of AP-2 and AP-3 complexes, indicating a role for both AP-2 and AP-3. VAMP7, as well as its t-SNAREs partners syntaxin 8 and Vti1, are co-immunoprecipitated with each of the medium subunits of the AP-1, AP-2, AP-3 and AP-4 complexes. This result supports the conclusion that VAMP7 directly interacts with both AP-2 and AP-3. It also raises the hypothesis of an interaction with AP-1 and AP-4. GFP-VAMP7 is retrieved from the endocytic pathway at and/or before the late post-lysosomal stage through an AP-independent mechanism.

Introduction

SNAREs are membrane proteins that play a key role in the fusion of intracellular membrane compartments [1,2]. SNAREs from the "v (vesicle)-SNARE" family are anchored to transport vesicles issued from a donor compartment, while SNAREs from the "t(target)-SNARE" family are located on target compartments to which the transport vesicles will fuse. Fusion is induced by formation of a complex between one v-SNARE and its two or three specific t-SNARE partners, in which α-helical domains or "SNARE motifs" from each SNARE assemble in a four α-helix coiled-coil bundle (review: [3]). According to the "SNARE hypothesis" [2], the localization of each SNARE to a specific compartment may thus be responsible for the specificity of membrane fusions within the cell. The mechanism involved in their sorting is therefore of primary importance. A random mutagenesis screen allowed the first identification of a dileucine sorting sequence in a t-SNARE, the yeast vacuolar t-SNARE Vam3p, homologous to the mammalian syntaxin 7 [4], and suggested interaction with the AP(clathrin-associated Adaptor Protein)-3 complex. Four tetrameric AP complexes (AP-1, AP-2, AP-3 and AP-4) control the transport of cargo proteins between the trans-Golgi network or the plasma

* *Corresponding author.* Fax: +33 4 38 78 44 99.
E-mail address: nelly.bennett@cea.fr (N. Bennett).

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membrane and the endosomal/lysosomal pathway (review: [5]). Although each complex plays a distinct role, all of them recognize the same two types of motifs (review: [6]): the "tyrosine motifs" (YXXΦ) and the "dileucine-based motifs" (two L(I) preceded by at least one acid residue usually at position −4). The critical role of a tyrosine motif was reported for the localization of mouse syntaxin 6 [7], and that of dileucine-based motifs for the localization of human syntaxin 7 and syntaxin 8 [8], and of mouse and rat VAMP4 [9,10]. GST-VAMP4 (but not GST-VAMP4 with a mutated dileucine motif) binds in vitro to AP-1, but not to AP-2 and AP-3 [10,11]. Another v-SNARE, human TI VAMP (VAMP7) was reported to bind to AP-3 from a two-hybrid screen and from immunoprecipitation experiments [12], but the domain responsible for the interaction was not precisely determined.

SNAREs are present in all eukaryotic cells, including yeast, plants, and mammals. Four homologues of mammalian SNAREs, the v-SNARE VAMP7 (vesicle associated membrane protein 7), and three t-SNAREs, syntaxin 7, syntaxin 8 and Vti1 (Vps10p tail interactor 1) were previously described in the soil amoeba *Dictyostelium discoideum* [13,14], and shown to participate in endosome fusion. The same four SNAREs participate in fusion of late endosomes to lysosomes in mammals [15], revealing a striking conservation of membrane fusion mechanisms from unicellular eukaryotes to mammals. *Dictyostelium* cells grown in liquid culture medium continually internalize fluid by macropinocytosis, and the cells are filled with large endocytic compartments of less than 1 to 5 μm diameter, which undergo multiple fusion events. This suggests that the SNAREs involved in endosome fusion might be present in unusually large amounts, and that *Dictyostelium* could be a powerful tool for studying the mechanisms of SNARE sorting. The medium subunits of AP-1, AP-2, AP-3 and AP-4 (μ1, μ2, μ3, μ4) and the large AP-1 γ subunit have been cloned in *Dictyostelium* [16,17]. All the other subunits are identified by homology with mammalian sequences in the *Dictyostelium* database (http://www.dictybase.org/). We investigated the possible role of AP complexes in VAMP7 sorting using different complementary approaches. The localization of GFP-VAMP7 (wild-type or with a mutation in a potential dileucine motif) was studied in the parental strain, and compared to the localization of GFP-VAMP7 in knockout cells for the gene of each of the AP complexes medium subunits (live cells). Interaction between VAMP7 and AP complexes was further investigated by immunoprecipitation of the four cell lines expressing the myc-tagged μ subunits.

Materials and methods

Cell culture, live cell fluorescence and immunofluorescence

Dictyostelium cells (strain: DH1.10 or AX2) were grown at 21 °C in a liquid axenic culture medium (HL5).

For live cell microscopy, cells were allowed to adhere to a glass coverslip for 30 min to 1 h, washed two or three times with PBS/5 buffer and observed with a Zeiss Axiovert 200M inverted microscope. The blue HL5 fluorescence emission was visualized using the Zeiss filter set 49 (DAPI) (excitation: 365 nm, beamsplitter 395 nm, emission: band-pass filter 445/50 nm). In some photos, HL5 fluorescence was artificially coloured in red instead of blue for better contrast with GFP. GFP was observed with the Zeiss filter set 44 (GFP). RFP (red fluorescent protein) and TRITC (tetramethylrhodamine isothiocyanate) were observed with the Zeiss filter set 15 (rhodamine).

For immunofluorescence analysis, cells were applied on a glass coverslip for 2 h, then fixed with 4% paraformaldehyde for 30 min, washed and permeabilized with methanol at −20 °C for 2 min. Cells were incubated with the antibody to p80 for 1 h, and then stained with an anti-mouse secondary antibody coupled to Alexa Fluor 568 (Molecular Probes, Leiden, Netherlands) for 30 min. Cells were observed by laser scanning confocal microscopy (Zeiss LSM 510).

Constructs

Full length VAMP7 (DDB0231535), syntaxin 7 (DDB0231537), syntaxin 8 (DDB0231533) and Vti1 (DDB0231536) were amplified by PCR from cDNA clones SSI250, SLH661, SSF583 and SSK661 respectively; full-length AP-3 δ subunit (DdAP3-D1, DDB 0234240) from cDNA clone SHF187 (dds34m21) and cDNA clone SLC526. GFP or myc-tag constructs were obtained by cloning in the *Dictyostelium* expression vector pDXD-3C [18]: GFP or a double myc-tag was inserted at the KpnI–SacI sites for fusion at the N-terminal end of the protein. Inserts were cloned at the BamHI–XhoI sites. Cells were transformed with pDXD-3C together with the pREP4 vector [18], and grown in the presence of G418 (20 μg/ml unless otherwise stated). Syntaxin 7 was also cloned at the BamH1–EcoRI sites in the pBsrH-mRFPmars vector downstream of RFP [19], and cells expressing RFP-syntaxin 7 were grown in the presence of 10 μg/ml Blasticidin. All constructs were expressed in the DH1.10 strain [20], a subclone of DH1 [21]. GFP-VAMP7 and GFP-VAMP7 [L35A] were also expressed in the AX2 strain. Cloning of μ1, μ2, μ3, μ4 [16], and knockout cells for the μ genes in the DH1.10 strain [17,22,23] were described previously.

Immunoprecipitation

Cells suspensions were shaken at 100 rpm in the presence (cells expressing myc-tagged μ subunits) or in the absence (control DH1.10 cells) of 60 μg/ml G418. For each cell type, 5×10^8 to 1.2×10^9 cells were collected by centrifugation (750 g, 5 min), and washed three times with 30 ml PBS/5 buffer.

(1) For identification of the SNAREs, the anti-myc antibody 9E10 was cross-linked to protein G-agarose beads (400 μg 9E10 per ml of beads) with dimethylsuberimidate (DMS) according to the manufacturer instructions. The cell pellets were suspended in 2 ml breaking buffer (100 mM NaCl, 50 mM phosphate buffer pH8.3, 2 mM $MgCl_2$, 2 mM EGTA, supplemented with protease inhibitors). The cells were broken with a cell cracker [24], and incubated with the water-insoluble thiol-cleavable cross-linker Dithiobis[succinimidylpropionate] (DSP) (2.5 mM diluted from a freshly prepared 200 mM solution in DMSO) for 40 min at 21 °C. The suspension was centrifuged for 30 min at 90 000 rpm in a Beckman TL100 centrifuge. The membrane fraction above the DNA transparent pellet was recovered by gentle pipeting with breaking buffer supplemented with 20 mM Tris pH8 (to quench the cross-linking reaction) and 2.5% Nonidet® P-40 (NP40). Solubilisation was achieved by rotating the tubes for 1 h at 4 °C, and the detergent extract was centrifuged again to remove non-solubilised particles. The cleared extract was incubated for 3 h with protein G-agarose beads (40 μl) to