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RNAi knockdown of parafusin inhibits the secretory pathway

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ABSTRACT

Several glycolytic enzymes and their isoforms have been found to be important in cell signaling unrelated to glycolysis. The involvement of parafusin (PFUS), a member of the phosphoglucomutase (PGM) superfamily with no phosphoglucomutase activity, in Ca^{2+}-dependent exocytosis has been controversial. This protein was first described in *Paramecium tetraurelia*, but is widely found. Earlier work showed that parafusin is a secretory vesicle scaffold component with unusual post-translational modifications (cyclic phosphorylation and phosphoglucosylation) coupled to stages in the exocytic process. Using RNAi, we demonstrate that parafusin synthesis can be reversibly blocked, with minor or no effect on other PGM isoforms. PFUS knockdown produces an inhibition of dense core secretory vesicle (DCSV) synthesis leading to an exo$^-$ phenotype. Although cell growth is unaffected, vesicle content is not packaged properly and no new DCSVs are formed. We conclude that PFUS and its orthologs are necessary for proper scaffold maturation. Because of this association, parafusin is an important signaling component for regulatory control of the secretory pathway.

Introduction

Parafusin (PFUS), first described in *Paramecium tetraurelia* (Satir et al., 1989) has a 51% sequence identity to phosphoglucomutase (PGM), with 4 insertions and a longer N-terminal as compared to rabbit muscle PGM (Subramanian et al., 1994). PFUS or its orthologs are present in most eukaryotic cells (Wyroba and Satir, 2000; Wyroba et al., 1995). PFUS and PRP1, its *Toxoplasma* ortholog (Matthiesen et al., 2001, 2003) have been shown to be phosphoglycoproteins and members of PGM superfamily with little or no PGM enzymatic activity (Satir et al., 1989; Subramanian et al., 1994; Andersen et al., 1994; Levin et al., 1999). Similarly other isoforms of PGM in various organisms have little PGM activity (Belkin and Burridge, 1995; Fu et al., 2000; Narayanan and Xu, 1997). Like certain isoforms of other glycolytic enzymes including GAPDH and aldolase (Daubenberger et al., 2003; Hessler et al., 1998; Rogalski-Wilk and Cohen, 1997), these non-enzymatic forms of PGM may be involved in cell signaling processes, rather than in glycolysis. PFUS and PRP1 function via post-translational modifications in membrane scaffolds of respectively, dense core secretory vesicles (DCSVs) in *Paramecium* (Zhao and Satir, 1998) and micronemes, the *Toxoplasma* secretory vesicles to facilitate Ca^{2+}-dependent processes related to regulated exocytosis (Matthiesen et al., 2001, 2003; Liu et al., 2009). When cells are stimulated to exocytose, in conjunction with a Ca^{2+}-dependent dephosphoglucosylation, PFUS/PRP1 dissociates from the vesicle scaffold and can no longer be specifically localized. PFUS/PRP1 re-associates with newly forming vesicles in the cytosol. Isolated DCSVs contain glycosylated PFUS in their scaffold (Liu et al., 2009).

Neither exocytosis, dephosphoglucosylation (Subramanian and Satir, 1992) or dissociation of the PFUS of the DCSV scaffold (Satir and Zhao, 1999) is seen in the temperature sensitive exo$^-$ mutant cells (nd9 – 27 °C) of *Paramecium* after stimulation at the non-permissive temperature. The nd9 gene product was found to be a novel protein with armadillo-like repeats that may play a role in possible protein–protein interactions, like the SNARE proteins (Froissard et al., 2001).

In a recent study using a heterologous system, Alexa-labeled His-PRP1 electroporated into living wild type *Paramecium* associated with DCSVs and co-localized with PFUS upon fixation. Upon exocytosis PRP1 dissociated from the DCSV only to re-associate as new organelles formed. Association of PRP1 with the vesicle scaffold could be blocked by site-specific mutagenesis of PRP1 (Liu et al., 2009). Taken together, these results suggest that PFUS is a significant component for proper scaffold formation around *Paramecium* DCSVs, and that after scaffold formation and organelle docking, the correct covalent modification of PFUS is essential for membrane fusion and exocytosis.

The importance of PFUS as both a component of the DCSV scaffold and a crucial component involved in exocytosis, but not with

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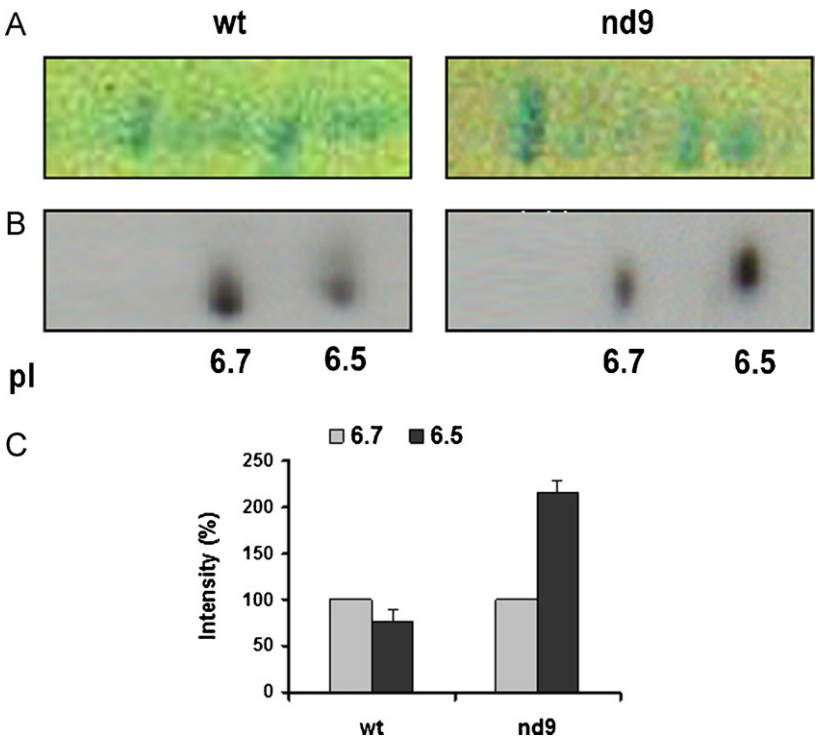

Fig. 1. 2D gel analysis of *Paramecium* PGM superfamily members. (A) Coomassie blue stained 2-D gel of immunoprecipitated PGM superfamily members with a pan-PGM antibody from wt exo$^+$ cells (left panel) in comparison to nd9 cells (right panel). Six ~63 kDa spots at pI 6.9–6.4 are present. Arrows indicate spots at pI 6.7 and 6.5. (B) Western blot: Western blot analysis of the above 2D gels for wt and nd9 cells probed with PFUS antibody reveals that only two of the six spots are recognized, arrows indicate the two spots at pI 6.7 and 6.5. (C) Intensity ratio of the spots at pI 6.5/6.7 shown in B. Please note that the intensity ratio ($n = 3$) of these spots is 0.75 for wt and 2.1 for nd9-27 cells.

glycolysis, has been a somewhat controversial idea. Now, RNAi methods (Bastin et al., 2001; Galvani and Sperling, 2002) have allowed us to establish that independent of other PGM superfamily members, PFUS knockdown produces an inhibition of DCSV synthesis leading to an exo$^-$ phenotype. These results support the conclusion that proper incorporation of PFUS into the scaffold is necessary for maintenance and maturation of the DCSVs and subsequently for Ca^{2+} regulated exocytosis. Orthologs of PFUS could serve similar roles in other cells. The results also suggest that, via even small changes or a post-translational modification, members of the PGM superfamily, can assume roles unrelated to glycolysis.

Materials and methods

Cells, media, and culture conditions

Axenic cultures of wt *Paramecium tetraurelia* or mutant nd9 (Sonneborn, 1974) and nd6 (Lefort-Tran et al., 1981) were grown at 27 °C in Cephalin-Fatty Acid (CFA) media (Satir and Bleyman, 1993) to early stationary phase, harvested (400 g, 30 s) and washed twice in Mg^{2+}-buffer (10 mM $MgCl_2$, 5 mM Tris–HCl, 1 mM KCl, pH 7.0).

Antibodies

Pan-PGM antibody is a rabbit polyclonal antibody raised against the isolated parafusin (Murtaugh et al., 1987). PFUS antibody was raised against the synthetic peptide (CDYEFEKHINLDQ) corresponding to the insertion-2 region of PFUS by Immuno-dynamics, Inc. T8 antibody was a monoclonal antibody raised against DCSV content T8, courtesy from Dr. Richard Allen, University of Hawaii.

Preparation of cell homogenate and immunoprecipitation

Collected and washed *Paramecium* cells were mechanically lysed on ice in glass homogenizer in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 2 μM leupeptin, 1.5 μM antipain, 0.5 mM PMSF, 0.012 TIU/ml aprotinin, 5 mM Na_3VO_4) Homogenate was centrifuged (12,000 × *g*, 5 min, 4 °C) twice and the supernatant (1 ml) was collected. Pan-PGM antibody (1:125) was added into the supernatant and the mixture was incubated at room temperature for 1 h. 50 μl of Protein G beads (Sigma, St. Louis, MO) were washed with lysis buffer and transferred into the homogenate–antibody mixture. After incubation (30 min, 4 °C on shaker), the beads were washed (with lysis buffer, 1500 g, 2 min, 4 °C) three times and collected.

Two-dimensional electrophoresis and Western blot

The proteins were resolved according to the manufacturer's manual for the Multiphor II horizontal two-dimensional gel electrophoresis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The first dimension (isoelectric focusing) was performed with Immobiline Dry Strip, pH 3–10 linear pH gradient and the second dimension (SDS-PAGE) was performed with ExcelGel SDS XL 12–14% Tris–glycine. The gel was either stained with Coomassie blue, or for western blot, transferred to nitrocellulose membrane (0.8 mA/cm^2 membrane, 1 h) by Semi-dry Blotting System (IMM-1, W.E.P. Co. Seattle, WA, USA). The membrane was blocked with 5% milk in washing buffer (10 mM Tris–HCl, 0.9% NaCl, pH 7.3) for 1 h, rinsed with 0.5% milk three times, and then incubated with 1:1000 PFUS antibody for 2 h. The membrane was washed three times with washing buffer and incubated for 1.5 h in 1:3000 HRP-conjugated goat anti-rabbit secondary antibody (Chemicon, Billerica, MA, USA). Membrane was then washed three times and developed with ECL (GE Healthcare, Little Chalfont, UK).