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A unique residue in rab3c determines the interaction with novel binding protein Zwint-1

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Abstract **Exocytic events are tightly regulated cellular processes in which rab GTPases and their interacting proteins perform an important function. We set out to identify new binding partners of rab3, which mediates regulated secretion events in specialized cells. We discovered Zwint-1 as a rab3 specific binding protein that bound preferentially to rab3c. The interaction depends on a critical residue in rab3c that determines the binding efficiency of Zwint-1, which is immaterial for interaction with rabphilin3a. Rab3c and Zwint-1 are expressed highly in brain and colocalized extensively in primary hippocampal neurons. We also found that SNAP25 bound to the same region in Zwint-1 as rab3c, suggesting a new role for the kinetochore protein Zwint-1 in presynaptic events that are regulated by rab3 and SNAP25.**

Keywords: Rab3; Zwint-1; Rabphilin3a; SNAP25

1. Introduction

Presynaptic neurons release neurotransmitters by synaptic vesicle exocytosis. This process is regulated by a number of conserved proteins including SNAREs, Sec1/munc18 family proteins and rab GTPases. Members of the rab3/rab27 subfamily are involved in exocytic events. The rab3a and rab3c isoforms are mainly expressed in brain, where they localize to synaptic vesicles [1]. Rab3a knockout mice exhibit abnormal regulation of synaptic vesicle exocytosis, but exocytosis itself is not impaired [2]. The rab3a deficient neurons show no aberrant synaptic morphology or composition except for a loss of the rab3 effector rabphilin3a [3]. Quadruple rab3 knockout mice die shortly after birth from respiratory failure, while triple rab3 knockout mice are viable, as long as rab3a is present [4]. These observations indicate a high degree of redundancy between the rab3 isoforms and suggest that rab3 is not part of the core membrane fusion machinery, but is involved in regulation of calcium induced fusion events.

Rab proteins are molecular switches that act through the interaction with effector proteins. Here, we report the identification of ZW10 interaction protein 1 (Zwint-1) as a novel rab3 binding protein, with rab3c as the strongest binding isoform. Zwint-1 is implicated in the regulation of faithful chromosomal segregation through binding to the kinetochore [5], but our results suggest an alternative function in a rab3 related process. We found that transfected Zwint-1 and rab3c extensively co-localized in synapses of primary hippocampal neurons. We also defined a crucial Ser residue in the switch I region of rab3c, responsible for the enhanced affinity of Zwint-1 with respect to the other rab3 isoforms.

2. Materials and methods

2.1. Nucleotide methods

pEF BOS FLAG-rab3a, rab3b, rab3c, rab3d and rab4 were a kind gift from Dr. Mitsunori Fukuda (Tohoku University, Japan). pEF BOS FLAG-rab3a P(49)S and rab3c S(57)P, pGEX 5X3 rab3c Q(83)L and rab3c T(36)N mutants were produced by Quick-change site directed mutagenesis (Stratagene). The cDNAs for the GST rab3 isoforms were cloned into the BamHI/EcoRI sites of pGEX 5X3. pGEX rab9 was obtained from Dr. Suzanne Pfeffer (Stanford University, USA) and the other rab pGEX plasmids have been described [6,7]. The cDNAs encoding Zwint-1 and SNAP25 were purchased from RZPD. Zwint-1 was cloned into the BamHI/EcoRI sites of pRK5-myc, and the XmaI/EcoRI sites of pGW1-GFP and pGW1-myc. SNAP25 was cloned into the SmaI site of pGW1-GFP. Zwint-1 truncations (amino acids 1–79, 1–95, 1–111, 1–128, 1–145, 1–179 and 1–226) were cloned into the BamHI/EcoRI sites of pRK5-myc. The peGFP-rabphilin3a construct was generously provided by Prof. Mathijs Verhage (Vrije Universiteit, Amsterdam) and peGFP ZW10 was given by Dr. Geert Kops (Universiteit Utrecht). Identity of synthetic DNA was verified by restriction analysis and dideoxy sequencing.

2.2. Antibodies

The following antibodies were purchased from indicated sources: anti-myc polyclonal (Upstate Cell Signalling); anti-myc monoclonal (Oncogene); anti-FLAG M2 monoclonal, anti-FLAG polyclonal and anti-FLAG M2 monoclonal conjugated to agarose (Sigma); anti-GFP polyclonal (Santa Cruz Biotechnology); anti-rab3c polyclonal (Bethyl laboratories); polyclonal anti-β-galactosidase (BD Biosciences); fluorescently labeled secondary antibodies (Jackson Laboratories).

2.3. Cell culture

COS-7 cells were grown in DMEM containing 10% fetal calf serum, antibiotics and 2 mM glutamine. Ins I cells were grown in RPMI 1640 containing 10% fetal calf serum, antibiotics, 2 mM glutamine, 1 mM

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sodium pyruvate and 50 μM β-mercaptoethanol. Primary rat hippocampal neuron isolation, culturing, transfection, labelling and imaging analysis were performed as described previously [8].

2.4. GST pulldown assay

Glutathione-*S*-transferase (GST)-rab proteins were produced at 30 °C in *Escherichia coli* BL21 (DE3) and immobilized on glutathione beads as per the vendors' instructions. Guanine nucleotide exchange was performed as described previously [7]. Ins I cells ($\sim 2.5 \times 10^9$) were homogenized in 10 ml buffer A (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT), spun for 1 h at 100 000 × g at 4 °C and the cytosol was incubated with immobilized rab protein for 2 h. The beads were washed and bound proteins were eluted as described [7]. Samples were resolved on SDS–PAGE and stained by coomassie brilliant blue. For some experiments we used transfected COS-7 cells that were lysed with buffer 1 (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.2% NP40 and 20 μM GMP-PNP). Lysates were spun 10 min at 16 000 g in a cooled micro centrifuge and supernatants were incubated with GST-rab beads for 2 h and washed four times with 1 ml buffer 1. Bound proteins were eluted by boiling in Laemmli sample buffer and analysed by Western blotting.

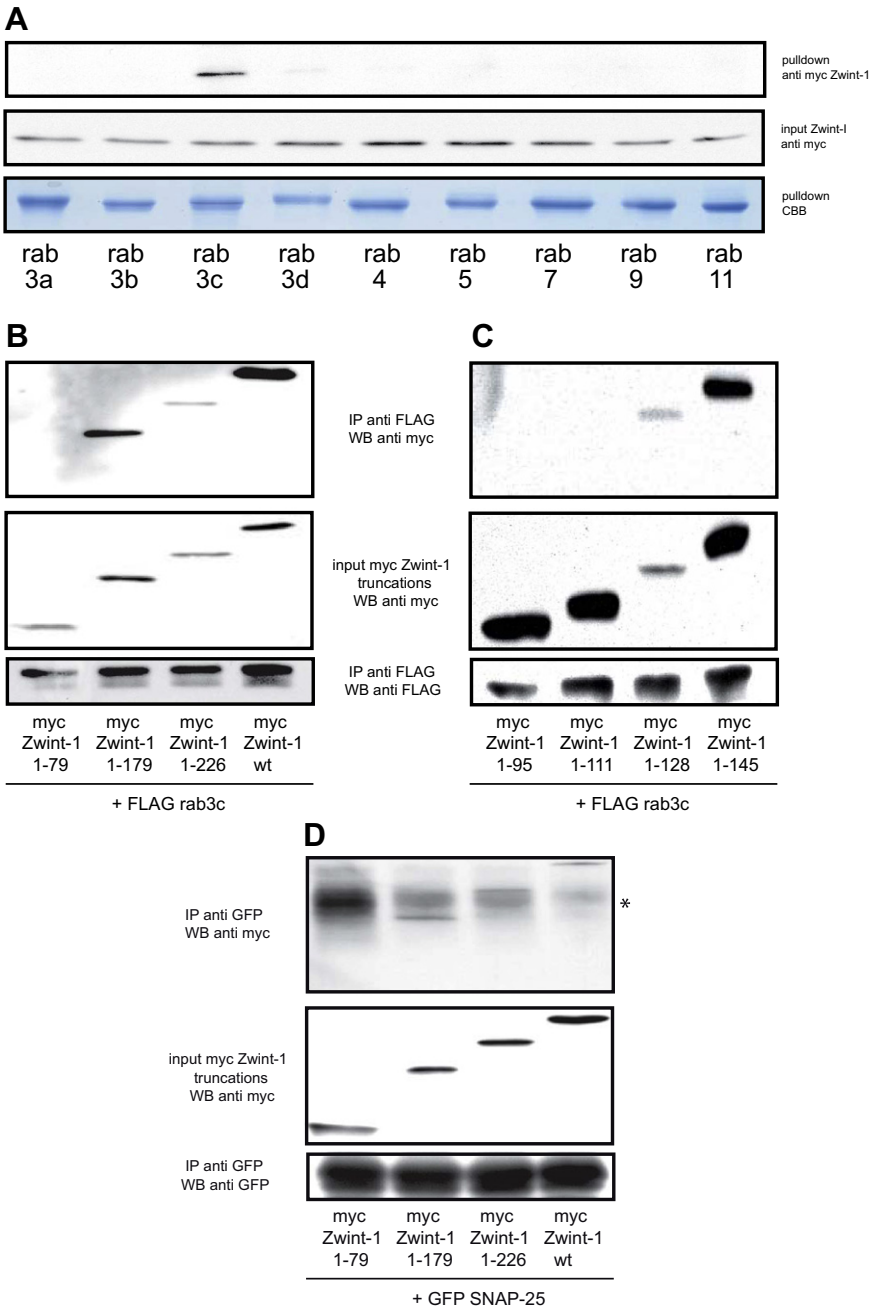

Fig. 1. Interaction between rab3 and Zwint-1 Lysates from COS-7 cells transfected with myc-Zwint-1 were subjected to a GST pulldown with GST-rab fusion proteins (input in bottom panel A). Bound proteins were eluted and analyzed by Western blot (A). COS-7 cells transfected with FLAG-rab3c (B and C) or GFP-SNAP-25 (D) and the indicated Zwint-1 constructs (input in middle panel) were subjected to FLAG (B and C) or GFP (D) immunoprecipitation. Bound proteins were analyzed by Western blot with antibodies against the myc, FLAG, and GFP epitopes (B–D). Asterisk indicates the IgG heavy chain.

2.5. Co-immunoprecipitation

COS-7 cells were transfected with bait and prey cDNAs using Lipofectamine 2000 (Invitrogen). After 20 h, cells were lysed for 10 min in buffer 1. Lysates were spun 10 min at 16000 × g in a cooled micro centrifuge and supernatants were incubated with 7.5 μl of washed FLAG M2 agarose beads for 35 min at 4 °C under continuous agitation. Beads were subsequently washed four times with buffer 1. Bound proteins were eluted by boiling in Laemmli sample buffer and analyzed by Western blotting.

3. Results and discussion

To identify new binding partners of members of the rab3/rab27 subfamily of rab GTPases a GST pulldown screen was performed. To this end we prepared cytosol from the pancreatic β-cell line Ins I and incubated this with GMP-PNP charged GST-rab beads. Bound proteins were eluted, resolved by SDS–PAGE and stained by coomassie brilliant blue. GST rab specific bands were subjected to tryptic digestion and LC–MS/MS [9]. Zwint-1 was identified by two peptides (QWVLQQK and AFEQLEAK) from one of the GST rab lanes. To investigate the rab specificity of the interaction we expressed myc-Zwint-1 in COS-7 cells. Lysates were incubated with GST-rab fusion proteins of members of the rab3/rab27 subfamily and a number of rab GTPases that regulate transport through endosomal routes. As shown in Fig. 1A we detected myc-Zwint-1 specifically in the GST-rab3c lane, with a minor fraction in the GST-rab3d lane, but none in the other GTPases that were tested.

To define the site of interaction of rab3c on Zwint-1 we performed binding assays by co-expressing FLAG-rab3c and myc-tagged Zwint-1 truncations in Cos-7 cells and subjecting the lysates to a FLAG immunoprecipitation followed Western blot analysis of the bound proteins. As shown in Fig. 1B, truncations 1–179 and 1–226 could be co-immunoprecipitated with FLAG-rab3c, while truncation 1–79 lost the ability to interact, indicating that the binding site is located between residues 79 and 179. This part of Zwint-1 coincides with a predicted

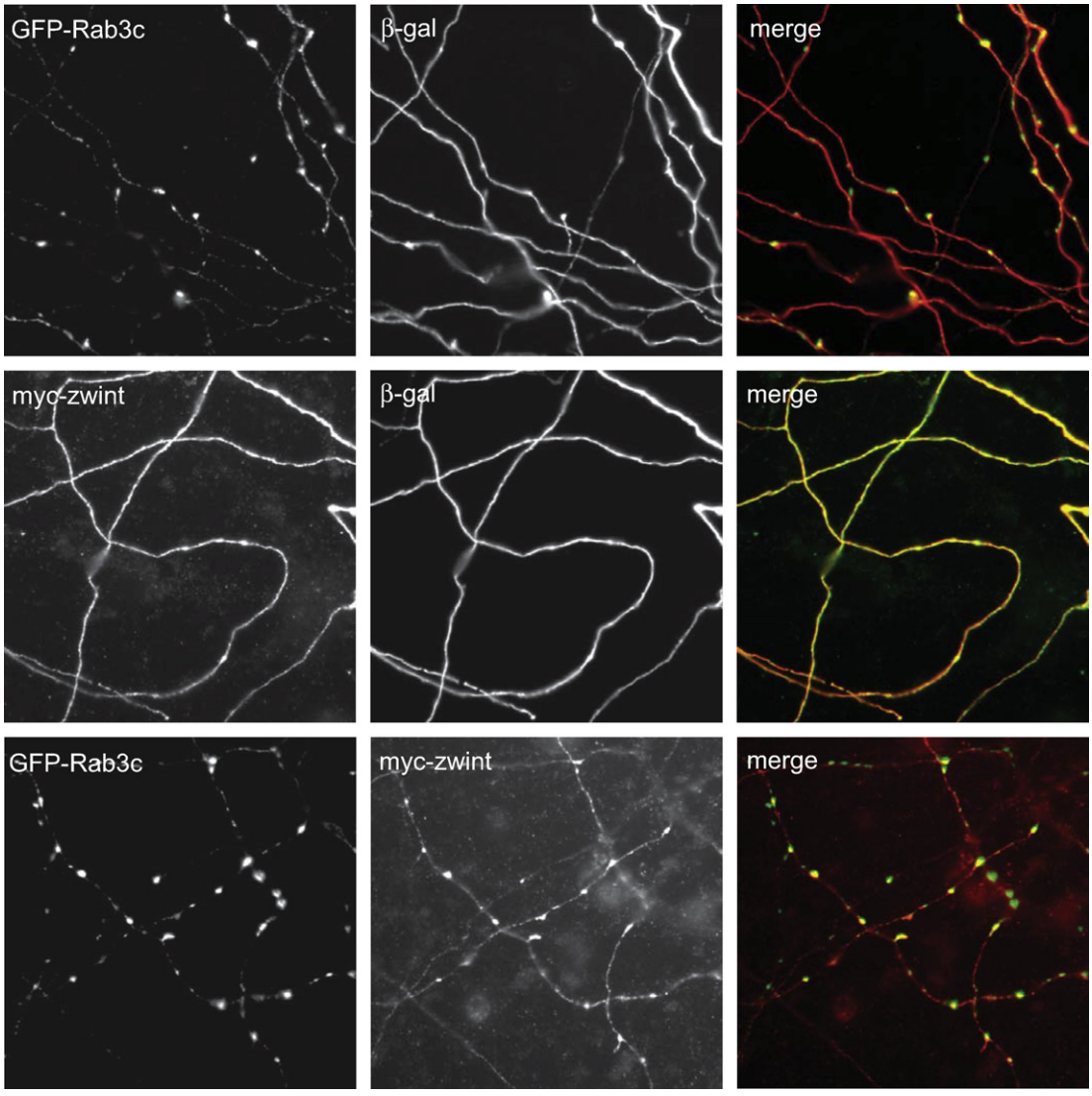

Fig. 2. Zwint-1 colocalizes with rab3c in primary hippocampal neurons Neurons from a rat hippocampus were isolated and transfected with GFP-rab3c in combination with β-galactosidase (top panels), myc-Zwint-1 in combination with β-galactosidase (middle panels) or a combination of myc-Zwint-1 and GFP-rab3c (bottom panels).

coiled-coil domain. Through an additional truncation analysis we narrowed down the binding domain to residues 111–128 (Fig. 1C). Zwint-1 otherwise known as SIP30 or scoilin also binds to SNAP25 [10]. To determine the binding domain for SNAP25 on Zwint-1, we co-expressed GFP-SNAP25 and myc-tagged Zwint-1 truncations in COS-7 cells and performed co-immunoprecipitations. As shown in Fig. 1D, SNAP25 binding to Zwint-1 depended on aa 79–179. The observation that both rab3c and SNAP25 bound to this region of Zwint-1 implicates Zwint-1 as a platform to integrate the activities of rab3c and SNAP25 protein networks in the presynaptic nerve terminal. Alternatively, SNAP25 and rab3c might compete for the same binding site on Zwint-1, and future work is needed to discriminate between these modalities. Importantly the interaction between with ZW10 occurs on a distinct part of Zwint-1 (not shown).

The interaction between rab3c and Zwint-1 prompted us to investigate their intracellular localisation. As both proteins are expressed highly in brain [5,10] we performed localisation studies in primary hippocampal neurons. The available antibodies against Zwint-1 did not allow reliable detection of the endogenous protein by immunofluorescence microscopy. Therefore, we co-transfected GFP-rab3c and myc-Zwint-1 or the cytoplasmic marker β-galactosidase together with GFP-rab3c or myc-Zwint-1 in hippocampal neurons (Fig. 2). With or without myc-Zwint-1, GFP-rab3c localized to punctae reminiscent of axonal synaptic structures, which corresponds with the reported localisation [11]. Single expression of myc-Zwint-1 resulted in a staining that was identical to that of co-transfected β-galactosidase. However, when GFP-rab3c and myc-Zwint-1 were co-expressed, the cytosolic labelling of Zwint-1 disappeared and most of the signal co-localized with

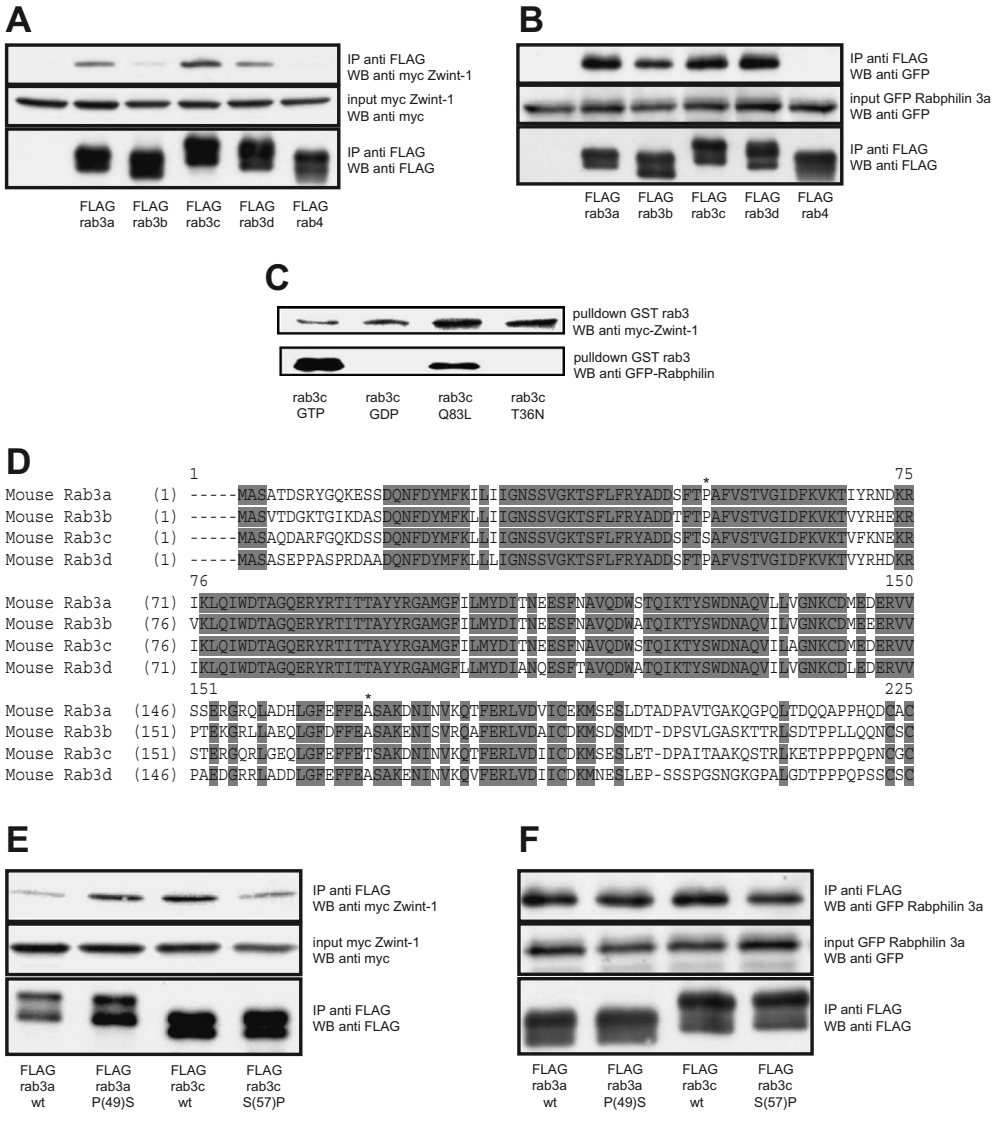

Fig. 3. Zwint-1 affinity is determined by a single residue in switch I region COS-7 cells transfected with the indicated FLAG-rab3 and myc-Zwint-1 constructs (A and E) or GFP-rabphilin3a (B and F), were subjected to FLAG immunoprecipitation. Bound proteins were analyzed by Western blot with antibodies against indicated epitopes. COS-7 cells expressing myc-Zwint-1 or GFP-rabphilin3a were subjected to a GST pulldown with indicated rab3c fusion protein (C). Sequences of mouse rab3 isoforms are aligned. Grey boxes illustrate conserved residues. Asterisks denote residues unique to rab3c versus the other rab3 isoforms (D).

GFP-rab3c in the synaptic punctae. These results showed that ectopically expressed rab3c recruited myc-Zwint-1 to synaptic structures, which possibly enhanced the wild type situation where endogenous rab3c might retain a small fraction of Zwint-1 in the synapses at any given point in time.

To explore the different binding behaviour of the rab3 isoforms (Fig. 1A) in vivo, we co-expressed myc-Zwint-1 and the FLAG-rab3 isoforms in COS-7 cells. The cells were lysed and subjected to a FLAG immunoprecipitation. In Fig. 3A we analysed the eluates from the FLAG beads and observed strongest binding to rab3c, followed by less robust signals on FLAG-rab3a and rab3d (46% and 47% of the rab3c signal, respectively) and the least binding to FLAG-rab3b (12% of the rab3c signal). To exclude that misfolding of rab3a, rab3b or rab3d reduced binding of these isoforms to Zwint-1, we repeated the binding experiment with the established rab3 effector rabphilin3a. In Fig. 3B, we showed that the rab3 isoforms bound to GFP-rabphilin3a with equal efficiencies, as was reported previously [12]. This showed that the enhanced binding of Zwint-1 to rab3c was not caused by improper folding of the other three FLAG-rab3 isoforms, but actually reflected bona fide differences in binding efficiency.

To establish whether or not the interaction depended on the nucleotide status of rab3c we performed a GST pulldown assay with GDP or GMP-PNP loaded rab3c and the active Q(83)L or inactive T(36)N mutants with lysates of COS-7 cells expressing myc-Zwint-1 or GFP-rabphilin3a. In Fig. 3C we observed that while rabphilin3a bound exclusively to activated rab3c, Zwint-1 interacted with both forms. These results showed that the binding of Zwint-1 to rab3c did not depend on the activation status of the rab, suggesting upstream regulation by for instance kinases or accessory proteins.

Since the rab3 isoforms bound with distinct efficiencies to Zwint-1, we set out to determine the mechanism for the observed differences. To this end we aligned the primary amino acid sequences of the rab3 isoforms (Fig. 3D). Interestingly, rab3c contains a Serine residue at position 57 that is completely conserved in rab3c from various species. In contrast, the other rab3 forms share a conserved Proline substitution at the corresponding position. This region of rab3 is part of the guanine nucleotide binding domain termed switch I. If Ser57 in rab3c is important for interactions with binding partners, we would expect to observe decreased Zwint-1 binding to a S(57)P-rab3c mutant compared to wild type rab3c. Conversely we anticipate increased binding of Zwint-1 to the P(49)S mutant of rab3a compared to wild type rab3a. We constructed the rab3c S(57)P and the rab3a P(49)S mutants, effectively swapping the switch I regions of these rab3 isoforms and used them in a co-immunoprecipitation assay with myc-Zwint-1. In Fig. 3E, in agreement with our hypothesis we observed a decrease in binding of the rab3c mutant compared to the wild type (from 100% to 54%) and the binding to the rab3a mutant was increased with respect to wild type rab3a (from 37% to 84% in relation to the binding to rab3c wild type). This indicated that the higher affinity binding of rab3c correlated with the sequence in the switch I region of rab3c. To asses whether this was specific for Zwint-1 we repeated the experiment with rabphilin3a instead of Zwint-1. As shown in Fig. 3F, we found that the wild type and mutant rab3 forms bound with the same efficiencies to GFP-rabphilin3a. This observation is in agreement with the structure of the binding domain of rabphilin3a and rab3a, where the sequence containing Pro47 is not part of the interaction surface [13]. These data suggest that the Ser57 in rab3c is specifically involved in either the interaction with Zwint-1 or in the generation of a structural element that is essential for the definition of binding efficiency.

In conclusion, we identified a critical residue in rab3c that specifically determined the binding affinity for Zwint-1. We also found that SNAP25 and rab3c bound to the same region of Zwint-1. Since neurons do not divide, these interactions suggest a novel role for the kinetochore protein Zwint-1 in presynaptic events that are regulated by rab3 and SNAP25. Indeed, the Zwint-1 binding partner ZW10 has been implicated in membrane transport processes during interphase [14], but a role for Zwint-1 was not investigated in this context.

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