Supplementary Materials

Deak et al., **Rabphilin Regulates SNARE-Dependent Re-Priming of Synaptic Vesicles for Fusion**

EXPANDED EXPERIMENTAL PROCEDURES

1. List of plasmids

1.1 GST expression vectors (all in pGEX-KG)

GST-Rph C2A, pGEX-KG-rat-rabphilin residues 377-528
GST-Rph C2B, pGEX-KG-rat-rabphilin residues 521-681
GST-Rph C2AB, pGEX-KG-rat-rabphilin residues 377-681
GST-full-length Rph, pGEX-KG-rat-rabphilin residues 1-681
GST-Syx, pGEX-KG-rat syntaxin residues 1-264
GST-SNAP-25A, pGEX-KG-rat-SNAP-25A full-length, residues 1-206
GST-Syb, pGEX-KG-bovine synaptobrevin 2, residues 1-96

Please note that our rabphilin construct is different from the initial Genbank sequence (U12571) in the following residues: T66A, residue 265-7 (GEQ) are deleted, and Q379E. However, these changes look correct when compared to the mouse and rat genome sequences. As a result, our rat rabphilin includes 681 amino acids. All of these plasmids were described previously in:

1.2 The new lentiviral plasmids.

pFUW-rat Rabphilin full-length (residue 1-681)
pFUW-rat Rabphilin without C2B (residue 1-535)

These are newly constructed plasmids produced as follows: GFP cDNA was removed from pFUGW plasmid (Lois C, et al., 2002. Science Vol 295: 868-872; Dittgen T, et al., 2004. PNAS Vol 101:18206-11) by BamH1 and EcoR1 digestion, and new multiple cloning sites (Xba1, EcoR1, BstB1, Nhe1, BamH1, Hpa1) were introduced after ligation of following oligo nucleotides, 5'-gatagaattcttcgaagctagcggatccgttaac-3' and 5'-aattgttaacggatccgctagcttcgaagaattc-3' to make pFUW plasmid. Full-length and C2B deleted rabphilin cDNA were subcloned into Xba1 and Nhe1 digested pFUW vector after PCR amplification using pfu polymerase.

These plasmids were described in the following papers:

Li, C., Takei, K., Geppert, M., Daniell, L., Stenius, K., Chapman, E.R., Jahn, R., De Camilli, P., and Südhof, T.C. (1994) *Synaptic targeting of rabphilin-3A, a synaptic vesicle Ca^{2+}/phospholipid-binding protein, depends on rab3A/3C*. Neuron **13**, 885-898.

Fykse, E.M., Li, C., and Südhof, T.C. (1995) *Phosphorylation of rabphilin-3A by Ca^{2+}/Calmodulin- and cAMP-dependent protein kinases in vitro.* J. Neurosci. **15**, 2385-2395.

McMahon, H.T. and Südhof, T.C. (1995) *Synaptic core complex of synaptobrevin, syntaxin, and SNAPS forms high affinity α-SNAP binding site.* J. Biol. Chem. **270**, 2213-2217.

Fernandez, I., Ubach, J., Dulubova, I., Zhang, X., Südhof, T.C., and Rizo, J. (1998) *Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A*. Cell **94**, 841-849.

2. List of antibodies

Protein	ID	Poly/Mono	Antigen	Dilution
GDI	81.2	Monoclonal		>2000
Rabphilin	84.2	monoclonal	Recombinant rabphilin	>2000
	76.2	monoclonal	Recombinant rabphilin	>2000
	I374	polyclonal	thrombin cleaved pGEX85-III	1000
	I375	polyclonal	uncleaved pGEX85-III	1000
	I731	polyclonal	pGEX85-8 (both C2 domains)	1000
	I734	polyclonal	pGEX85-4 (N-terminal half)	1000
	N321	polyclonal	pGEX-NP2-1a (Doc2)	1000
	P588	polyclonal	peptide	1000
	U344	polyclonal	phospho-Rabphilin peptide	1000
Syntaxin	HPC-1	monoclonal		>2000
	I378	polyclonal	Syntaxin 1A GST-fusion	1000
	I379	polyclonal	Syntaxin 1A GST-fusion	1000
	P941	polyclonal	Peptide	1000
	T3748	polyclonal	Stx A peptide	1000
	U6250	polyclonal	a.a. 1-264	1000
Munc-18	Comm.	monoclonal		1000
	J370	polyclonal	pMAL-Munc18	1000
	J371	polyclonal	purified Munc18	1000
	K329	polyclonal	GST-Munc-18	1000
	P592	polyclonal	peptide	1000
SNAP-25	71.1	monoclonal		>2000
	71.2	monoclonal		>2000
	77.1	monoclonal		>2000
	77.2	monoclonal		>2000
	I733	polyclonal	GST-SNAP-25	1000
	P913	polyclonal	peptide	1000
Synaptobrevin	69.1	monoclonal		>5000
	D204	polyclonal	peptide: CSTGVPSGSSAAT	1000
	D212	polyclonal	peptide: CSTGVPSGSSAAT	1000
	P939	polyclonal	peptide	1000
Synaptophysin	7.1	monoclonal		>2000
	7.2	monoclonal		>2000
	7.3	monoclonal		>2000
	7.4	monoclonal		>2000
	K831	polyclonal	GYGDAGYGQGPGGYGPQDSYGPQGGYQPD	1000
	L164	polyclonal	CEVEFEYPFRLHQ	1000
	L166	polyclonal	p38-3 peptide	1000
	P580	polyclonal	peptide	1000
	P611	polyclonal	GYGPQGDYGQQGYGQQGAPTSFSNQM	1000
	U2658	polyclonal	TyrP peptide: CGPQGG[Y-Phos]QPD	1000
	U2659	polyclonal	TyrP peptide: CGPQGG[Y-Phos]QPD	1000
	U2660	polyclonal	Syp-IIIP peptide	1000
	Y941	polyclonal	p37-1 synaptoporin peptide:	1000

			CEFGQQPSGPTSFNNQI (Syp-2)	
Rab3	42.1	monoclonal		>2000
	42.2	monoclonal		>2000
	P583	polyclonal	peptide	1000
	T957	polyclonal	Rab3a peptide: CASATDARYGQKES	1000
	V757	polyclonal	purified Rab3a	1000
	U953	polyclonal	Rab3b	1000
	U954	polyclonal	Rab3b	1000
	P180	polyclonal	Rab3c	1000
	P181	polyclonal	Ra3c	

These antibodies were previously described in the following papers:

Fykse, E.M., Takei, K., Walch-Solimena, C., Geppert, M., Jahn, R., De Camilli, P., and Südhof, T.C. (1993) *Relative properties and localizations of synaptic vesicle protein isoforms: The case of the synaptophysins.* J. Neurosci. **13**, 4997-5007.

Fykse, E.M., Li, C., and Südhof, T.C. (1995) *Phosphorylation of rabphilin-3A by Ca^{2+}/Calmodulin- and cAMP-dependent protein kinases in vitro.* J. Neurosci. **15**, 2385-2395.

Johnston, P.A., Archer, B.T., III, Robinson, K., Mignery, G.A., Jahn, R. and Südhof, T.C. (1991) *Rab3A attachment to the synaptic vesicle membrane mediated by a conserved polyisoprenylated carboxy-terminal sequence*. Neuron **7**, 101-109.

Li, C., Takei, K., Geppert, M., Daniell, L., Stenius, K., Chapman, E.R., Jahn, R., De Camilli, P., and Südhof, T.C. (1994) *Synaptic targeting of rabphilin-3A, a synaptic vesicle Ca^{2+}/phospholipid-binding protein, depends on rab3A/3C*. Neuron **13**, 885-898.

Verhage, M.., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M., Geuze, H., and Südhof, T.C. (2000) *Synaptic assembly of the brain in the absence of neurotransmitter secretion*. Science **287**, 864-869.

Khvotchev, M. and Südhof, T.C. (2004) *Stimulus-Dependent Dynamic Homo- and Heterodimerization of Synaptobrevin/VAMP and Synaptophysin.* Biochemistry **43**, 15037-15043.

Schlüter, O., Schmitz, F., Jahn, R., Rosenmund, C., and Südhof, T.C. (2004) *A complete genetic analysis of neuronal Rab3 function.* J. Neurosci. **24**, 6629-6637.

Chandra, S., Fornai, F., Kwon, H.-B., Yazdani, U., Atasoy, D., Liu, X., Hammer, R.E., Battaglia, G., German, D.C., Castillo, P.E., and Südhof, T.C. (2004) *Double Knockout Mice for α- and β-Synucleins: Effect on Synaptic Functions.* Proc. Natl. Acad. Sci. USA **101**, 14966-14971.

3. Immunoprecipitations

Rat brain synaptosomes were prepared by Ficoll gradient centrifugation and solubilized by resuspension at 1 g protein/l in 20 mM HEPES-KOH pH 7.4, 0.14 M KCl, 2 mM $MgCl_2$, 1 % Triton X-100, 1 mM EGTA, 0.2 mM PMSF, 1 g/l pepstatin, 1 g/l aprotinin, and either 1 mM GDP or 0.1 mM GTPγS, with or without 1.5 mM $CaCl_2$. After rotation at 4 ºC for 1 hr, insoluble material was removed by centrifugation (100,000 x g for 20 min), and the supernatant (1 ml/reaction) was incubated for 2-4 hrs at 4 ºC with 15 µl anti-rabphilin serum and the indicated additions of Ca^{2+}, EGTA, GDP, or GTPγS. Afterwards, a 50% slurry of protein G-Sepharose (35 µl) was added for a further 30 min incubation period, beads were washed three times in the same buffer, and bound proteins were solubilized in 50 µl SDS-PAGE buffer, subjected to SDS-PAGE, and analysed by immunoblotting as indicated. To control for the efficiency of rabphilin immunoprecipitation,

immunoprecipitations were immunoblotted with the rabphilin antibodies described above and with a rabphilin monoclonal antibody (Cl76.2).

4. GST-pulldown experiments

One frozen rat brain (~1.6 g; Pelfreeze) was homogenized in 10 ml of 50 mM HEPES-NaOH pH 7.2, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, protease inhibitor cocktail (Roche), and 4 mM EGTA, and then solubilized in 1% Triton X-100 by rotation for 1 hr at 4 ºC. Insoluble material was pelleted by high-speed centrifugation (100,000g), and the supernatant (300 µl) was added to glutathione beads containing GST fusion proteins (~15 µg GST-fusion protein), and incubated overnight at 4 ºC with or without 1 mM free Ca^{2+}. After incubation, the beads were washed six times with the binding buffer, and bound proteins were eluted by SDS-sample buffer. To pull down recombinant SNAP-25, 1 µg of SNAP-25 was added to different GST-rabphilin proteins (~5 µg).

5. NMR spectroscopy

^{15}N-labeled proteins were extensively equilibrated in 40 mM acetate pH 6.1, 150 mM sodium chloride, 0.02% sodium azide, 5% deuterium oxide and either 200 mM EDTA or 10 mM Ca^{2+} chloride, and examined at a concentration of 75 µM (1:1 molar ratio) in all experiments except for a final experiment in the presence of Ca^{2+} containing 150 µM SNAP-25 (1:2 molar ratio). ^{1}H-^{15}N two-dimensional HSQC experiments were acquired in an INOVA600 (Varian) spectrometer at 31.5 ºC (acquisition times = 1-10 hrs) with a sensitivity-enhanced pulse sequence that incorporates pulsed field gradients and a water flip back pulse, using spectral widths of 8000 and 1336 Hz for the F2 and F1 dimensions, respectively (Ubach et al., 1999). The data (2x100 FIDs of 768 complex points each) were obtained after Fourier transformation and removal of the aliphatic part on F2; matrices of 512x512 real points were recorded. The spectra were used to calculate cross-peak shifts as a function of SNAP-25 using a weighted distance criterium (Farmer et al., 1996). We defined $\Delta\delta_{ppm} = [(^{HN}\Delta\delta_{ppm})^2 + [(^{N}\Delta\delta_{ppm} \times 0.17)^2]^{1/2}$, where $^{HN}\Delta\delta_{ppm}$ and $^{N}\Delta\delta_{ppm}$ are the ^{1}H and ^{15}N shifts in ppm. Values of $\Delta\delta_{pp}$ larger than 0.04 ppm were considered significant, and are colored in red in Fig. 3.

REFERENCES for supplementary methods

Farmer BT 2nd, Constantine KL, Goldfarb V, Friedrichs MS, Wittekind M, Yanchunas J Jr, Robertson JG, Mueller L (1996) Localizing the NADP+ binding site on the MurB enzyme by NMR. *Nat Struct Biol* **3**: 995-997.

Ubach J, Garcia J, Nittler MP, Südhof TC, Rizo J (1999) The C_2B-domain of rabphilin: structural variations in a janus-faced domain. *Nature Cell Biol* **1**: 106-112