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Supporting information for

Peroxisome protein import: A complex journey.

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Materials and Methods

Recombinant Protein Preparation:

PEX5C was prepared as previously described [1].

PEX5(1-304) (termed PEX5N) was prepared from the full-length PEX5 expression vector pET28b-*At*PEX5 [1] *via* restriction digest with *Hind*III to introduce four cuts in the PEX5 DNA. The largest fragment, corresponding to pET28b-PEX5(bp1-1159), was isolated by gel extraction, re-ligated with T4 DNA ligase, transformed into XL-10 Gold and confirmed *via* sequencing. A frame shift brought the C-terminal haxahistidine tag into the reading frame.

A colony of pET28b-PEX5N transformed BL21 STAR™ (DE3)/pRARE2 was inoculated into LB (5 mL) and incubated at 37 °C, 220 rpm for 16 h. This culture was diluted 1:500 into LB media (1 L) and incubated at 37 °C, 220 rpm until an OD_{600} 0.4-0.6 was reached, then induced with IPTG (1 mM) and incubated for a further 20 h. Cell pellets were obtained *via* centrifugation (4,000 *g,* 5 min, 4 °C) from 1 L of culture. The cell pellet was re-suspended in 25 mL chilled lysis buffer (NaH_2PO_4 (50 mM), NaCl (300 mM), Glycerol (15 % v/v), β-metacapethanol (10 mM), pH 8.0) supplemented with 1 × Complete EDTA-free protease inhibitors (Roche) and lysed *via* sonication using a Soniprep 150 with a 10 mm diameter probe (8 Hz, 30 s on/off cycles, 10 min). The lysate was then cleared *via* centrifugation (20,000 *g,* 30 min, 4 °C). A slurry of Ni-NTA resin (1 mL) (Qiagen) in 50% EtOH was loaded into a column (ca. 0.5 mL Ni-NTA agarose beads) and washed with water (5 mL) and lysis buffer (3 × 5 mL). The resin was re-suspended in the supernatant fraction and incubated for 1 h at 4 °C with constant agitation. The mixture was loaded into a column and the supernatant allowed to flow through. The resin was washed with lysis buffer containing 50 mM imidazole (3 × 5 mL). His_6-PEX5N-His_6 was eluted in lysis buffer containing 250 mM imidazole (6 × 0.5 mL).

Co-Immunoprecipitation:

PEX5 immune serum was raised against amino acids ^{231}K-^{450}D of *A. thaliana* PEX5 in rabbit, and was a kind gift from Dr Makoto Hayashi [2].

PEX5N and PEX5C (3 μM final concentration) were combined on ice in a final volume of 250 μL in PBS. The mixtures were incubated with anti-PEX5 N-terminal antibody (1 μL) with gentle agitation at 4 °C for 1 h. Protein A coupled beads (25 μL) (Sigma) were added and the mixture gently agitated at 4 °C for 1 h. Beads were separated by centrifugation (1000 *g,* 2 min, 4 °C), washed with PBS (3 × 1 mL), and bound proteins eluted from the resin in 2 ×

SDS PAGE sample buffer (1 × 25 μL). PEX5 constructs were detected by anti-polyhistidine immunoblotting as previously described [1].

		0	10
MGSSHHHHHH	SSGLVPRGSH	**MAMRDLVNGG**	**AACAVPGSSS**
20	30	40	50
SSNPLGALTN	**ALLGSSSKTQ**	**ERLKEIPNAN**	**RSGPRPQFYS**
60	70	80	90
EDQQIRSLPG	**SELDQPLLQP**	**GAQGSEFFRG**	**FRSVDQNGLG**
100	110	120	130
AAWDEVQQGG	**PMPPTGPMFE**	**PVQPTFEGPP**	**QRVLSNFLHS**
140	150	160	170
FVESSRGGIP	**FRPAPVPVLG**	**LSQSDKQCIR**	**DRSSIMARHF**
180	190	200	210
FADRGEEFIN	**SQVNALLSSL**	**DIDDGIQARG**	**HVPGRFRELD**
220	230	240	250
DYWNESQAVV	**KPNLHPADNW**	**AAEFNQHGMD**	**HGGPDSWVQS**
260	270	280	290
FEQQHGVNGW	**ATEFEQGQSQ**	**LMSSQMRSMD**	**MQNIAAMEQT**
300			
RKLAAALEHH	HHHH		

Figure 1. Amino acid sequence of His_6-PEX5N-His_6.

Domains are indicated as: hexahistidine (green), PEX5 sequence (**bold**), WX_3F/Y PEX14 binding motifs (highlight blue).

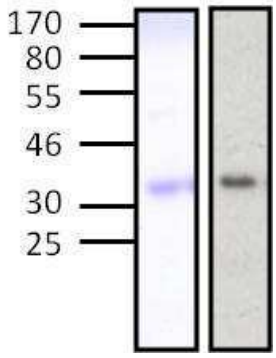

Figure 2. Analysis of purified PEX5N.

Left panel Coomassie stain, right panel α-polyhistidine immunoblot. His_6-PEX5N-His_6 (MW 36.7 kDa) is the band at ~35 kDa. Ladder denotes MW in kDa.

References:

1 Lanyon-Hogg, T., Hooper, J., Gunn, S., Warriner, S. L. and Baker, A. (2014) PEX14 binding to Arabidopsis PEX5 has differential effects on PTS1 and PTS2 cargo occupancy of the receptor. *FEBS Lett.* **588**, 2223-2229

2 Nito, K., Hayashi, M. and Nishimura, M. (2002) Direct interaction and determination of binding domains among peroxisomal import factors in *Arabidopsis thaliana*. *Plant Cell Physiol.* **43**, 355-366