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Purification, crystallization and preliminary X-ray diffraction analysis of the yeast Sec12Δp protein, a guanine nucleotide-exchange factor involved in vesicle transport

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Sec12 is a guanine nucleotide-exchange factor (GEF) of the GTP-binding protein Sar1. Its GEF activity on Sar1 makes it a key element in vesicle budding from the endoplasmic reticulum to the Golgi apparatus in yeast. Sec12 is an integral membrane glycoprotein of 70 kDa. A 38.5 kDa N-cytoplasmic domain (Sec12Δp) has been expressed in *Saccharomyces cerevisiae* and in *Escherichia coli*, purified to homogeneity and crystallized. Two crystal forms were obtained. Crystal form I belongs to space group $P6_2/P6_4$, with unit-cell parameters $a = b = 191.7$, $c = 53.3$ Å, $\gamma = 120^\circ$, and diffracts to 2.6 Å resolution. Crystal form II belongs to space group $P1$, with unit-cell parameters $a = 52.6$, $b = 53.0$, $c = 116.8$ Å, $\alpha = 98.0$, $\beta = 97.4$, $\gamma = 93.4^\circ$, and diffracts to 2.0 Å resolution.

1. Introduction

Sec12 is an integral membrane glycoprotein of 70 kDa required for the formation of a vesicular-transport intermediate in protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus in yeast (Nakano *et al.*, 1988). This protein is a type II membrane protein, with a prominent 40 kDa NH_2-terminal cytoplasmic domain, a single hydrophobic transmembrane domain and a COOH-terminal domain localized in the ER lumen (d'Enfert *et al.*, 1991). Whereas the membrane-anchored cytoplasmic domain is essential for protein transport using COP-II vesicles, the C-terminal domain is dispensable. The cytoplasmic domain is the site of interaction with other components required for the formation of transport vesicles derived from the ER, such as Sec13p, Sec16p and Sec23p (Kaiser & Schekman, 1990) and Sar1p, a small GTP-binding protein of 21 kDa (Nakano & Muramatsu, 1989). Sec12p acts as the guanine nucleotide-exchange factor (GEF) of Sar1p GTPase, which converts Sar1p from the inactive GDP form to the active GTP form (Barlowe & Schekman, 1993). Barlowe & Schekman (1993) have expressed the cytoplasmic domain of Sec12 (Sec12Δp) in yeast. This expression results in a soluble 38.5 kDa protein which is still capable of catalyzing Sar1p guanine-nucleotide exchange.

As a first step towards understanding the structural mechanism of vesicle budding from the ER, we report here the crystallization and preliminary crystallographic studies of the N-cytoplasmic domain of Sec12 (Sec12Δp).

2. Experimental results and discussion

2.1. Expression and purification

Sec12Δp was expressed in *S. cerevisiae* YPH499 cells transformed with pSKY2-1. This plasmid is controlled by a GAP promoter. Cells were grown in MCD medium (0.67% yeast nitrogen base, 0.5% vitamin assay casamino acid) containing 2% glucose and 20 mg l^{-1} each of tryptophan and adenine. Yeast cells were grown at 303 K for 24 h to an optical density of around 1.5 at 600 nm. Cells were diluted into 50 volumes of the same medium and grown for 20 h at 303 K. The purification protocol is based on the method of Barlowe & Schekman (1995). 4 l of Sec12Δp-overexpressing cells were harvested by centrifugation at 5000*g* for 20 min, washed twice with cold water and resuspended in 80 ml ice-cold buffer *A* (25 m*M* K HEPES pH 6.8, 0.25 *M* sorbitol and 1 m*M* magnesium acetate) containing 10 m*M* potassium acetate and 1 m*M* phenylmethylsulfonyl fluoride. Cells were lysed with glass beads and unbroken cells were removed by centrifugation at 20 000*g* for 1 h. Soluble lysate was loaded onto a 100 ml DEAE-Sephacel (Pharmacia) column equilibrated with buffer *A* containing 10 m*M* potassium acetate. Protein was eluted from the column using a linear gradient of 10–400 m*M* potassium acetate in buffer *A*. The peak fractions containing immunoreactive Sec12Δp were pooled, diluted into two volumes of buffer *A* and loaded onto a 15 ml CM-Sepharose CL-6B column (Pharmacia) equilibrated with buffer *A* containing 100 m*M* potassium acetate. Protein was eluted with a

linear gradient of 0.1–1 *M* potassium acetate in buffer *A*. Fractions containing pure Sec12Δp were identified by SDS–PAGE, immunoblotted with anti-Sec12Δp antibody and then pooled. An Amicon ultrafiltration unit was used to concentrate the protein and to exchange the buffer to buffer *A* containing 150 m*M* potassium acetate. The protein concentration was determined by UV spectroscopy at 280 nm using a calculated extinction coefficient of 0.479. Typically, 400 µg of 95% pure Sec12Δp was obtained from 4 l of culture.

With the intention of obtaining a higher yield from the expression and of obtaining a selenomethionine-substituted protein, Sec12Δp was expressed in *E. coli* as a His-tagged protein. Two constructs have been made to express Sec12Δp with an N-terminal His tag (His-Sec12ΔpNB) or with a C-terminal His tag (His-Sec12ΔpNC). *E. coli* BL21(DE3)pLysS cells carrying the recombinant plasmid were grown at 310 K in Luria broth medium supplemented with 50 µg ml^{-1} ampicillin. When the absorbance at 600 nm of the culture reached a value of 0.6, expression was induced by the addition of 0.5 m*M* IPTG. The cells were harvested 3 h after induction by centrifugation at 5000*g* for 15 min. The pellet from 1 l of culture was resuspended in 25 ml buffer *B* (50 m*M* sodium phosphate pH 7.5, 500 m*M* NaCl, 10 m*M* imidazole) and disrupted by sonication. Unbroken cells were removed by centrifugation at 20 000*g* for 30 min. The crude extract was applied to a 5 ml HiTrap column (Pharmacia) charged with nickel and pre-equilibrated with buffer *B*. The column was washed with ten column volumes of buffer *B* and ten column volumes of buffer *B* containing 50 m*M* imidazole. Protein was eluted from the column using a 30 ml linear gradient of 50–700 m*M* imidazole. Fractions were analyzed by SDS–PAGE and the peak fractions were combined. The sample buffer was changed to buffer *A* containing 10 m*M* potassium acetate using an Amicon ultrafiltration unit. The subsequent chromatography steps were carried out on an FPLC apparatus. The sample was loaded on a MonoS (Pharmacia) column pre-equilibrated with buffer *A* containing 100 m*M* potassium acetate; the protein was eluted from the column using a linear gradient of 0.1–1 *M* potassium acetate. Fractions containing His-Sec12Δp were concentrated to 1 ml using an Amicon ultrafiltration unit. To obtain a homogenous pool of protein for crystallization trials, the sample was loaded onto a Superdex 200 (Pharmacia) gel-filtration column equilibrated in buffer *A* containing 150 m*M* potassium acetate. Fractions containing pure His-Sec12Δp were identified by SDS–PAGE and pooled. This protocol yields approximately 7 mg of 95% pure protein from 1 l of culture.

To produce selenomethionine-labelled Sec12Δp, *E. coli* BL21(DE3)pLysS cells carrying the recombinant plasmid were grown in minimal medium M9, which was supplemented 30 min before induction with selenomethionine and an ample amount of other amino acids known to inhibit methionine biosynthesis (Van Duyne *et al.*, 1993). The purification of selenomethionine His-Sec12Δp was identical to that of unlabelled Sec12Δp and yielded 3 mg of pure protein from 1 l of culture. The selenomethionine incorporation was verified by mass spectrometry.

2.2. Crystallization

Crystals were initially grown by the hanging-drop technique using Hampton Crystal Screens (Jancarik & Kim, 1991). The protein used for crystallization trials was concentrated to 15–20 mg ml^{-1} using Microcon 30 filter units in buffer *A* containing 150 m*M* potassium acetate. 2 µl protein solution was mixed with 2 µl of the crystallization solution on a cover slip, which was then sealed with silicon oil over a well containing 1000 µl of the respective crystallization solution. The first crystalline precipitates were obtained at 277 K in solution 4 (0.1 *M* Tris–HCl pH 8.5, 2 *M* ammonium sulfate) of Hampton Research Crystal Screen I with the protein expressed in *S. cerevisiae*. Further optimization of the crystallization conditions led to diffraction-quality crystals using ammonium sulfate as a precipitant. In the vapour-diffusion setup, the reservoir was composed of 0.1 *M* K HEPES pH 6.8, 2.4–2.6 *M* ammonium sulfate. These first crystals (form I) are single needle-like crystals with approximate dimensions of 50 × 50 × 200 µm and grew within four weeks at 277 K (Fig. 1). Similar crystals were obtained under the same conditions with the N-terminal and the C-terminal His-Sec12Δp protein expressed in *E. coli*.

Another set of crystals (form II) were obtained at 293 K in solution 17 (0.085 *M* Tris–HCl pH 8.5, 25.5% PEG 4000, 15% glycerol) of Hampton CryoScreen with the C-terminal His-Sec12Δp protein (His-Sec12ΔpNC) expressed in *E. coli* (Fig. 1). These crystals are plates and the largest examples attained dimensions of 400 × 400 × 20 µm.

2.3. Data collection and analysis

Diffraction data were collected from both forms at 100 K using synchrotron radiation (ID14-EH3 and ID14-EH1 beamlines at the ESRF, France). A MAR CCD 165 detector and X-ray radiation of 0.936 or 0.934 Å wavelength were used. In order to collect low-temperature data, the form I crystals were soaked in a cryoprotectant solution containing 22% glycerol and the reservoir solution. The form II crystals were directly mounted on a cryoloop and flash-frozen *in situ* in the nitrogen stream at 100 K.

Initial indexing, data-collection strategies and processing were carried out using the program *MOSFLM* (Leslie, 1992) and subsequent data reduction was carried out using the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

Crystals of form I grow in the hexagonal space group $P6_2$ (or its enantiomorph $P6_4$), with unit-cell parameters $a = b = 191.7$,

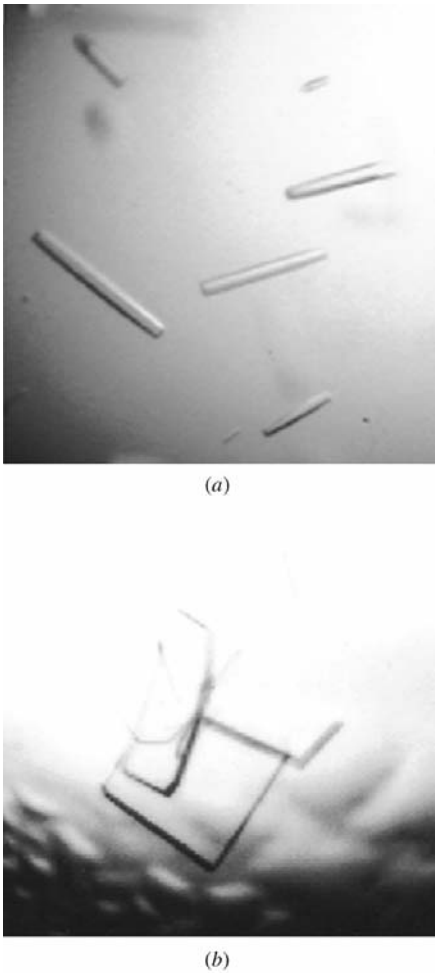

Figure 1
Crystallization of yeast Sec12Δp. (*a*) Form I: hexagonal crystals of yeast Sec12Δp (50 × 50 × 200 µm); (*b*) form II: plates of His-Sec12ΔpNC obtained with the C-terminal His-Sec12Δp protein expressed in *E. coli* (400 × 400 × 20 µm).

Table 1
X-ray diffraction data of the Sec12Δp crystals.

Values in parentheses correspond to the outer resolution shell (2.95–2.8 Å for form I, 2.07–1.96 Å for form II).

Crystals	Form I: crystal of Sec12Δp expressed in yeast	Form II: native crystal of His-Sec12ΔpNC expressed in *E. coli*
Experimental conditions		
X-ray source	ESRF ID14-EH3	ESRF ID14-EH1
Wavelength (Å)	0.936	0.934
Sample temperature (K)	100	100
Detector	MAR CCD	MAR CCD
Crystal parameters		
Resolution (Å)	15–2.8	29.1–1.96
Unit-cell parameters		
a, b, c (Å)	191.7, 191.7, 53.3	52.6 53.0 116.8
α, β, γ (°)	90.0, 90.0, 120	98.0, 97.4, 93.4
Space group	$P6_2$ or $P6_4$	$P1$
Mosaicity (°)	0.4	0.65
Solvent content (%)	66.5	70.3
Data processing		
No. reflections used	78397	153397
No. unique reflections	25462	47763
$I/\sigma(I)$	10.1 (2.8)	10.5 (6.7)
Completeness (%)	97.5 (96.2)	94.3 (94.3)
Multiplicity	2.9 (2.7)	3.0 (2.9)
R_{sym}† (%)	5.3 (0.1)	13.4 (25.2)

† $R_{sym} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I_i(hkl)\rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

c = 53.3 Å, $\gamma = 120°$, and diffract to 2.6 Å. A complete native data set was only collected to 2.8 Å as the intensity of the diffraction spots in the outer shell (2.6–2.8 Å) decayed quickly during the experiment. Because of the weak diffracting power of the crystal, it was exposed in three passes of 30 s for each degree of oscillation. A total of 78 397 reflections were measured and reduced to 25 462 unique reflections, which corresponds to a completeness of 96.2% to 2.8 Å resolution. The overall R_{sym} value is 5.3% (see Table 1 for a summary of the statistics). The V_M value of 3.6 Å^3 Da^{-1} (Matthews, 1968) is consistent with the presence of two molecules (of 38.5 kDa each) in the asymmetric unit and corresponds to a solvent content of 66.5%. Self-rotation calculations in the 180° κ section with intensity data between 15 and 3.0 Å resolution using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) confirm the existence of non-crystallographic twofold symmetry.

Crystal form II belongs to primitive space group $P1$, with unit-cell parameters a = 52.6, b = 53.0, c = 116.8 Å, α = 98.0, β = 97.4, γ = 93.4°. These crystals diffract to 2.0 Å resolution. The crystal was exposed for 5 s per degree of oscillation. 153 397 reflections were measured and reduced to 47 763 unique reflections, which corresponds to a completeness of 94.3% to 2.0 Å resolution. The overall R_{sym} value is 13.4% (see Table 1). The V_M value of 4.14 Å^3 Da^{-1} is consistent with the presence of two molecules (of 38.5 kDa each) in the asymmetric unit and corresponds to a solvent content of 70.3%.

The His-Sec12Δp protein expressed in *E. coli* has been used to prepare selenomethionyl-substituted protein for multi-wavelength anomalous diffraction (MAD) experiments (Hendrickson, 1991).

The crystal structure determination of Sec12Δp using the MAD method is now under way.

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