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Elucidation of molecular functions of human tumor suppressor protein 101F6 by reconstitution into phospholipid bilayer nanodiscs

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ABSTRACT

A candidate human tumor suppressor gene 101F6 product was expressed successfully in *Pichia pastoris* yeast cells. The purified 101F6 protein was successfully incorporated into phospholipid bilayer nanodiscs with different sizes by employing two reconstitution methods; self-assembly and reconstitution into the preformed empty nanodisc. The reconstituted 101F6 protein could be reduced with ascorbate quickly and was very stable even at ambient temperatures.

Keywords: *cytochrome b_{561}; nanodisc; ascorbate; membrane protein; electron transfer; apoptosis*

INTRODUCTION

The 101F6 gene in human 3p21.3 region codes for a heme-containing protein 101F6 that is a member of cytochrome b_{561} family being expressed in ER membranes with a six-α-helices transmembrane structure and is predicted to function as a tumor suppressor (Ji *et al.* 2002). Indeed the expression of 101F6 protein inhibits tumor cell growth in 3p21.3-deficient non small cell lung cancer cells *in vitro* and *in vivo* (Ohtani *et al.* 2007). However, the molecular mechanism of 101F6-mediated tumor suppression is unknown. In the present study, we conducted reconstitution of purified 101F6 protein into phospholipid bilayer nanodiscs (Ritchie *et al.* 2009) with three different sizes in diameter to elucidate the molecular mechanism of 101F6-mediated tumor suppression by determining the details of transmembrane electron transfer from ascorbate.

EXPERIMENTAL

Expression and purification of 101F6 protein were conducted by employing alcohol-assimilating yeast *Pichia pastoris* cells and pPICZB-101F6-His8 plasmid (Recuenco *et al.* 2013a, Recuenco *et al.* 2013b). In brief, human 101F6 gene was incorporated into the *Pichia* genome and successful transformants were selected by zeocin-resistance screening. Purification of 101F6 protein was conducted by using a Ni-NTA-Sepharose affinity column. Better purification results were obtained when DDM instead of β-OG was used as a solubilizing detergent. To conduct functional studies, purified 101F6 protein was incorporated into phospholipid bilayer nanodiscs, which were assembled from phospholipids DMPC

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and a class of membrane scaffold proteins (MSP1D1, MSP1D1ΔH5, MSP1E3D1). Three kinds of MSPs were each produced by *E. coli* BL21(DE3) strain after transformation with pMSP1D1, pMSP1E3D1, or pET28a-MSP1D1ΔH5, respectively and were purified. Reconstitution was conducted for the self-assembly of 101F6 protein into nanodiscs with three different sizes in diameter (MSP1D1ΔH5, 9.0 nm; MSP1D1, 9.7 nm; MSP1E3D1, 12.1 nm) by mixing at an optimized ratio of MSP : lipids : 101F6 protein.

RESULTS AND DISCUSSION

We searched optimized conditions for the self-assembly of 101F6 protein into nanodiscs by changing the mixing ratio of MSP : lipids : protein. We found that a ratio of 2:160:1 was the best for the reconstitution of MSP1D1ΔH5-101F6. A typical result for the reconstitution and its analyses in a molecular level are shown in Fig.1. Similarly, nanodisc complexes could be formed for other sizes such as MSP1D1 (intermediate size) and MSP1E3D1 (larger size).

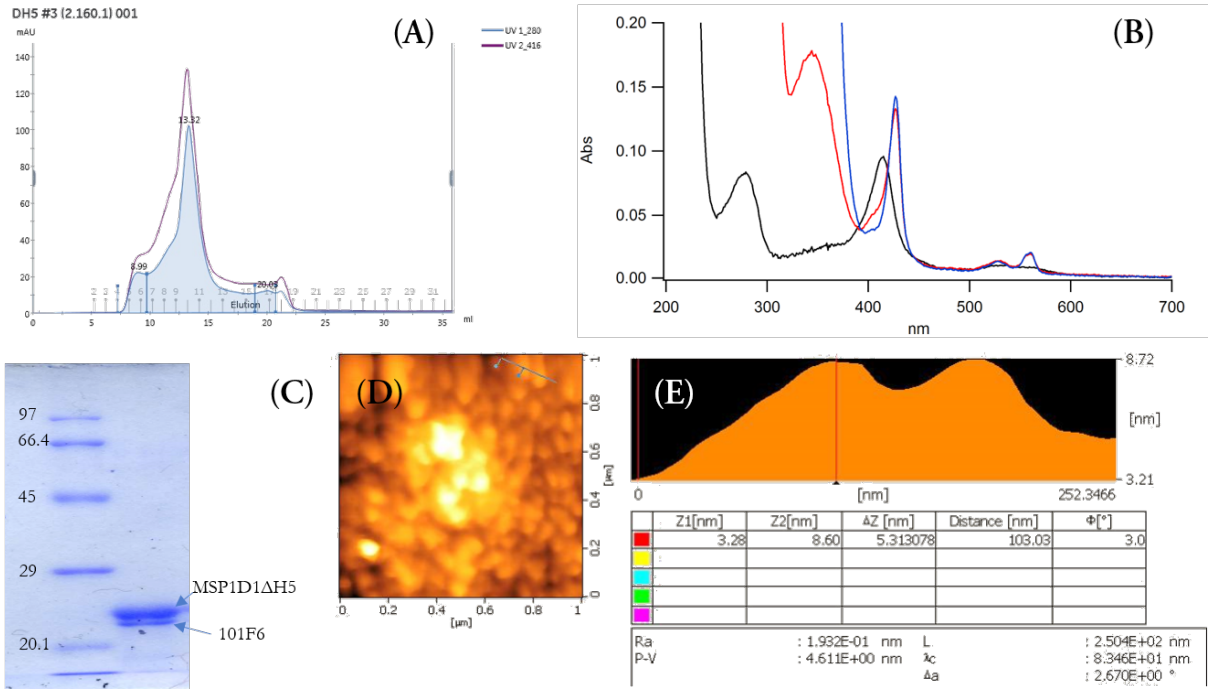

Figure 1. Successful reconstitution of 101F6 protein into nanodisc with a small size (MSP1D1ΔH5). (A) Elution profiles from gel filtration chromatography using a Superdex 200 HR 10/300 column (with AKTA chromatography system; GE Healthcare); the peak at 13.3 mL corresponding to the MSP1D1ΔH5-101F6 nanodisc complex; (B) Spectral analyses of MSP1D1ΔH5-101F6 nanodisc after the gel filtration; (C)SDS-PAGE (CBB staining) analysis of the purified nanodisc complex (lane 2); (D)An AFM image of the purified MSP1D1ΔH5-101F6 nanodisc (~1 μM) on freshly cleaved mica and (E) its dissection analysis along a line indicated in (D). Images were measured and analyzed by Nano Navi Probe Station (SII Nanotechnology).

The reconstituted 101F6 protein could be reduced with ascorbate very quickly (Fig. 1B; red line). Based on the calculations of absorbance at 280 nm, 427 nm and 561 nm (blue line, dithionite-reduced) of the nanodisc complex, we could roughly estimate a ratio between empty nanodisc and the 101F6-nanodisc complex by assuming only monomeric or dimeric 101F6 protein was incorporated (based on the estimation of the nanodisc size, only monomeric or dimeric 101F6 protein can be accommodated). The calculation indicated that most of nanodiscs exist as empty (~52%). Since the self-assembly of the 101F6-nanodisc complex is a time-consuming and low-yield method, we conducted an alternative way, in which empty nanodiscs were first prepared and purified and, then, were used for the reconstitution of purified 101F6 protein. This new method was found to be very effective, particularly for a larger size nanodisc MSP1E3D1, with the empty population decreased to 13%. The reconstituted 101F6 protein by this method was found to be very stable even at room temperatures for 1~2 weeks, indicating a significant increase in stability in native-like lipid bilayer environments. To directly observe the structure of these bilayer disks, we performed atomic force microscopy under aqueous buffer conditions. As shown in Fig. 1D and E, homogenous population of structures with 5~10 nm in height or diameter were observed, being consistent with the expected structure for the MSP1D1ΔH5-101F6 nanodisc

complex. Thus, present study established that the 101F6-nanodisc complex can provide a unique and versatile medium for studying the detailed molecular mechanism of 101F6-mediated tumor suppression.

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