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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 b₅₆₁; 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 b₅₆₁ 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, MSP1D1∆H5, MSP1E3D1). Three kinds of MSPs were each produced by *E. coli* BL21(DE3) strain after transformation with pMSP1D1, pMSP1E3D1, or pET28a-MSP1D1∆H5, respectively and were purified. Reconstitution was conducted for the self-assembly of 101F6 protein into nanodisks with three different sizes in diameter (MSP1D1∆H5, 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 nanodisks 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 MSP1D1∆H5-101F6. A typical result for the reconstitution and its analyses in a molecular level are shown in Fig.1. Similarly, nanodisk complexes could be formed for other sizes such as MSP1D1 (intermediate size) and MSP1E3D1 (larger size).

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 nanodisk complex, we could roughly estimate a ratio between empty nanodisk and the 101F6-nanodisk complex by assuming only monomeric or dimeric 101F6 protein was incorporated (based on the estimation of the nanodisk size, only monomeric or dimeric 101F6 protein can be accommodated). The calculation indicated that most of nanodisks exist as empty (~52%). Since the self-assembly of the 101F6-nanodisk complex is a time-consuming and low-yield method, we conducted an alternative way, in which empty nanodisks 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 nanodisk 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 MSP1D1∆H5-101F6 nanodisk.
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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