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Properties of Human Tumor Suppressor 101F6 Protein as a Cytochrome b_{561} and Its Preliminary Crystallization Trials

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1. Introduction

Identification of the physiological roles and elucidation of the molecular mechanisms involving tumor suppressor genes and their gene products are important for a more comprehensive understanding of cancer pathogenesis. Since the Knudson's statistical studies on retinoblastoma, neuroblastoma, and pheochromocytoma, which led to the conclusion that the occurrence of these tumors fits a two-mutation model (Knudson, 1971; Knudson & Strong, 1972), it became recognized that there were some genes that function to inhibit tumor development. The model stated that tumorigenesis results when there are genetic alterations such as deletions and mutations in both alleles of a gene in a cell (Knudson, 1971; Knudson & Strong, 1972). A tumor suppressor gene may have one or more functions related to cell division and differentiation, extracellular communication, tissue formation or senescence (Hollingsworth & Lee, 1991).

Several regions of the human chromosome 3 have been identified as susceptible sites for homozygous deletions and mutations that may lead to inactivation of one or more tumor suppressor genes. A particular tumor suppressor gene candidate *101F6* is located within a narrow 630-kb region on chromosome 3p.21.3, called LUCA (lung cancer region) (Lerman & Minna, 2000; Zabarovsky et al., 2002). Interestingly, the 101F6 protein is expressed in normal lung bronchial epithelial cells and fibroblasts but is lost in most lung cancers (Ohtani et al., 2007). Previous studies have shown that forced expression of the *101F6* gene *via* adenoviral vector-mediated gene transfer (Ji et al., 2002) or *via* nanoparticle injection (Ohtani et al., 2007) caused the inhibition of tumor growth in non-small cell lung cancer cells *in vitro* and *in vivo*. The treated cancer cells were also found to accumulate ascorbate (AsA) when incubated in a medium containing AsA (Ohtani et al., 2007). Apoptosis and autophagy of the cancer cells were reportedly to be enhanced by the treatment and were postulated to be caused by the synergistic action of the *101F6* gene and AsA though the mechanism of the action is still not clear (Ohtani et al., 2007).

The human *101F6* gene was found to encode a protein consisting of 222 amino acids and was predicted to be a member of the cytochrome b_{561} protein family (Tsubaki et al., 2005). Proteins such as adrenal cytochrome b_{561} (Tsubaki et al., 1997), duodenal cytochrome b_{561} (Mckie et al., 2001), and stromal cell-derived receptor 2 (Vargas et al., 2003) that were classified under this family have a common "b561 core domain" consisting of four transmembrane α-helices that have four totally conserved His residues for the binding of two heme *b* groups (Okuyama et al., 1998; Tsubaki et al., 2005). The adrenal cytochrome b_{561}, as a classic representative of this family, is a highly hydrophobic protein, consisting of six transmembrane α-helices with a molecular mass of 28 kDa and is located in the secretory vesicle membranes of adrenal chromaffin cells. This protein is involved in a transmembrane electron transfer reaction from cytosolic AsA to intravesicular monodehydroascorbate (MDA) radical that replenishes reducing equivalents to maintain physiological levels of AsA inside the vesicles (Kobayashi et al., 1998; Seike et al., 2003). AsA is an essential water-soluble vitamin that maintains the activity of copper-containing enzymes such as dopamine β-hydroxylase and peptidylglycine α-amidating monooxygenase by providing electrons to the copper center of the enzyme (Prigge et al., 2000) and to keep the intravesicular side in reduced state to protect otherwise very labile catecholamines. For efficient electron transfer, adrenal cytochrome b_{561} contain a putative AsA-binding motif on the cytosolic side close to the cytosolic heme center and a putative MDA-radical binding motif on the intravesicular side close to the intravesicular heme center, respectively (Okuyama et al., 1998). Such motifs were found to be conserved in other subfamilies of cytochrome b_{561}, including duodenal cytochrome b_{561} and plant cytochrome b_{561} (Tsubaki et al., 2005).

Comparative analysis on the amino acid sequences of seven subfamilies of the cytochrome b_{561} protein family showed that 101F6 protein does not contain the MDA-radical binding motif while the AsA-binding motif was significantly modified ("modified motif 1") (Tsubaki et al., 2005). These results suggested that redox active biofactor(s) other than AsA or MDA radical might be responsible as redox mediators of the 101F6 protein (Tsubaki et al., 2005). It is very intriguing to consider that the 101F6 protein has a role for transmembrane redox signal transduction *via* this unknown redox-linked activity. Therefore, the "modified motif 1" may be a primary candidate for conducting such transmembrane redox reactions (Tsubaki et al., 2005). Thus, clarification of the properties and three-dimensional structure of the 101F6 protein is highly necessary in understanding the role of this transmembrane protein as a tumor suppressor and as a controlling factor in human lung cancer development.

Although the cytochrome b_{561} protein family has a large numbers of its members (human tissues contain 6 members), none of them has ever been successfully crystallized for analysis by X-ray diffraction. Though we have attempted the crystallization for various members of the cytochrome b_{561} family, we have been limited by the amount of samples for more extensive screenings. A major barrier to crystallizing membrane proteins from higher eukaryotes (animals and plants) is the inability to purify sufficient amounts of non-denatured active proteins for conducting the crystallization trials. Indeed, except for the classic cytochrome b_{561} protein from bovine adrenal chromaffin vesicles, purification of cytochrome b_{561} from native tissues has been found to be almost impossible. Further, heterologous expression systems for the membrane protein including the members of the cytochrome b_{561} family, by employing the prokaryote *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, the insect *Sf9* cells, and mammalian cells, showed limited success based on the evaluation of their final qualities and/or quantities. Recently, the methylotrophic yeast, *Pichia pastoris*, has proven to be a very useful system to express and purify milligram

quantities of membrane proteins (Abramson et al., 2003; Huang et al., 2003; Jiang et al., 2003). However, instances about its use as a host for the expression of mammalian membrane proteins was limited (Long et al., 2005).
We have previously reported about the functional expression, purification and characterization of recombinant human 101F6 protein, which was expressed as a poly-histidine tagged protein in methylotrophic yeast *Pichia pastoris* (Recuenco et al., 2009). The purified 101F6 protein exhibited characteristic properties as a member of the cytochrome b_{561} protein family, particularly with regards to spectral characteristics and electron transfer activities with AsA and MDA radical. In this paper, we want to present our optimized protocol for the human 101F6 protein expression and purification. Further, we have succeeded in the crystallization of recombinant human 101F6 protein for the first time. We also present preliminary results on the quality of the human 101F6 protein crystals.

2. Materials and methods

2.1 The *Pichia pastoris* expression system

In our present study, we employed the *Pichia pastoris* expression system (*Pichia pastoris* GS115 cells and a pPICZB vector; from Invitrogen Corp., Tokyo, Japan) for the successful expression of human *101F6* gene. As a single-celled microorganism, yeast *Pichia pastoris* is easy to manipulate and is, therefore, very suitable for culture. However, it is a eukaryote and capable of many of post-translation modifications onto the heterologously expressed proteins such as proteolytic processing, folding, disufide bond formation, and glycosylation, which are performed by higher eukaryotic cells. Most importantly, *Pichia pastoris* cell is a very suitable host for the expression of membrane proteins, particularly for integral membrane proteins, because of its eukaryotic nature (*e.g.*, presence of membranes within the cytosolic milieu).
It was proposed that, compared to insect cells (*e.g.*, *Sf9* cell) or other mammalian cultured cells, *Pichia pastoris* cells are much easier to handle, can be grown at lower cost, and can be expressed quicker in a large scale (Asada et al., 2011). Such successful examples for expressing eukaryotic membrane proteins were reported previously (Weiß et al., 1998; Wetterholm et al., 2008; Nakanishi et al., 2009a; Nakanishi et al., 2009b; Alisio & Mueckler, 2010; Ostuni et al., 2010; Mizutani et al., 2011).

2.2 Construction of the expression plasmid pPICZB-101F6-His_8

Procedure for a molecular cloning of the human 101F6 gene was described previously (Recuenco et al., 2009). Construction of the expression plasmid, pPICZB-101F6-His_8, was described previously (Recuenco et al., 2009). Briefly, the thrombin-specific sequence followed by the eight-histidine residue-tag (QPSALVPRGSSAHHHHHHHH; the underline indicates the thrombin-specific sequence) was introduced at the C-terminus of human 101F6 protein, resulting in a total of 240 aa residue-long with a molecular mass of 25996.9 Da. An eight-histidine residue-tag sequence, instead of a usual six-histidine residue-tag, was added to provide a stronger binding affinity towards Ni-NTA affinity column. Such a poly-histidine-residue tag was employed for the expression of mammalian glucose transporter (eight-histidine residue) (Alisio & Mueckler, 2010) and G-protein coupled receptors (deca-histidine residue) (Yurugi-Kobayashi et al., 2009). Introduction of the tag-sequences at the C-terminus of human 101F6 protein was chosen with considerations about the increase in length of the C-terminal part and its successful protein expression in the ER membranes of *Pichia pastoris* cells.

2.3 Expression and purification of the human wild-type 101F6 protein

The pPICZB-101F6-His_8 plasmids obtained from the transformed *E. coli* cells were purified and linearized using *Pme* I and were used for the transformation of *Pichia pastoris* GS115 competent cells according to EasyComp™ transformation protocol (Invitrogen Corp., Tokyo, Japan). Selection was done by plating onto YPDS medium containing 400 μg/mL Zeocin (Invitrogen Corp., Tokyo, Japan), a bleomycin-like compound that kills cells by introducing lethal double-strand breaks in chromosomal DNA. In our screening process, the concentration of Zeocin was increased four times higher than the recommended concentration of 100 μg/mL, to obtain Zeocin hyper-resistant transformants that would have multicopy clones. The Zeocin-resistant protein (the product of the *Sh ble* gene in the pPICZB vector) confers resistance to the transformed cells stoichiometrically, not enzymatically, by binding to and inactivating the drug. Therefore, such transformants may ultimately result in an increase in the level of heterologous 101F6-His_8 protein production (Romanos et al., 1998).

Single colonies with a hyper Zeocin-resistant activity from the YPDS-Zeocin plates were inoculated into 1000-mL Erlenmeyer flasks with a baffled bottom containing 250 mL BMGY media (2% glycerol). During the usual induction procedure of such transformants by the addition of methanol (final 2%) as described previously (Recuenco et al., 2009), the color of the medium become reddish, indicating a successful expression of a holo-form of the 101F6 protein in the *Pichia pastoris* cells. After harvesting the induced cells, microsomal fractions were prepared in the presence of protease inhibitor cocktail (Protease Inhibitor Cocktail for General Use (100X); Nacalai Tesque, Kyoto, Japan). Then, the cytochrome b_{561} content based on the absorption at 561 nm was calculated. Typical results showed a total yield of 600~1000 nmoles of cytochrome b_{561} in microsomal fraction obtained from a 250-mL culture after 96 h of incubation (Table 1).

Solubilization of microsomal membrane fraction was conducted with β-octyl glucoside (2%)(Anatrace, Maumee, Ohio, USA). All the following steps were conducted at 4°C or on ice to avoid the formation of aggregates. Further, to obtain a better yield, we skipped the step of DEAE-Sepharose column chromatography, which was included in the original purification procedure (Recuenco et al., 2009). Thus, the solubilized membrane proteins from the microsomal fraction were directly applied to a pre-packed Ni-NTA-Sepharose (GE Healthcare Japan. Tokyo, Japan) column equilibrated with a buffer containing 300 mM NaCl and 10 mM imidazole. Removal of interfering proteins was achieved by washing the column with the buffers containing 20 mM imidazole and 50 mM imidazole. Then, the 101F6-His_8 protein was eluted with the buffer containing 300 mM imidazole. The purified sample was promptly desalted with PD-10 column (GE Healthcare Japan, Tokyo, Japan) equilibrated with 50 mM phosphate buffer containing 1 % β-octyl glucoside and 10 % glycerol.

3. Results

3.1 Properties of the purified 101F6-His_8 protein

Table 1 shows a typical example for the protein yield and the cytochrome b_{561} content at all the purification steps. From a 250-mL culture, which provides about 16-20 grams of wet cell bodies expressing the 101F6-His_8 protein with an approximate cytochrome b_{561} content of 800~900 nmoles, it was possible to purify about 0.60 mg of the recombinant human 101F6-His_8 protein with the cytochrome b_{561} content of 180 nmoles, with a purification fold of 56

from the stage of microsomes to the stage of the final desalting. Since the cytochrome b_{561} content at the stage of microsomes would include a considerable amount of other *b*-type cytochromes, such as cytochrome bc_1 complex, the actual yield of the recombinant 101F6-His_8 protein would be much better. Then, the purified 101F6-His_8 protein was evaluated by SDS-PAGE, visible absorption spectroscopy, MALDI-TOF mass spectrometory, redox titration, heme content analysis, in comparison with those of classic chromaffin granule (CG) (*i.e.*, chromaffine vesicle) cytochrome b_{561}.

SDS-PAGE analysis on the purified 101F6-His_8 protein showed a single protein band with its estimated molecular weight around 26 kDa. Achievement of the highly purified sample by a single column chromatography step as in our present study might be possible by the better binding affinity of the 101F6-His_8 protein towards Ni-NTA-Sepharose column, by the introduction of eight-histidine residue-tag at the C-terminus (Alisio & Mueckler, 2010). Occasionally we found a dimer (or trimer) form of the 101F6-His_8 protein monomer upon the SDS-PAGE analysis. We found that such a formation of the dimer (or trimer) form was not due to a disulfide bond formation between the monomers and could be avoided without the heat treatment of the purified sample before the analysis. The great tendency to form aggregates of the 101F6-His_8 protein during the purification steps is likely to be related to a spontaneous formation of the dimer (or trimer) and might be the intrinsic nature of the cytochrome b_{561} protein family more or less (Apps et al., 1984; Duong et al., 1984; Liu et al., 2011).

Visible absorption spectra of the purified 101F6-His_8 protein were analyzed in the region from 700 to 340 nm. The purified 101F6-His_8 protein exhibited typical spectra characteristics as a member of the cytochrome b_{561} family (Tsubaki et al., 1997). A strong Soret band at 414 nm and weak Q bands between 500-600 nm were observed for the oxidized form. Addition of sodium dithionite produced the fully-reduced form with a Soret band at 427 nm and resolved Q bands at 529 (β-band) and at 561 nm (α-band) (Figure 1). For the heme content analysis, the purified 101F6-H_8 protein was converted to a pyridine hemochrome by the addition of pyridine and NaOH according to the method of (Fuhrhop & Smith, 1975) as modified with (Berry & Trumpower, 1987). An extinction coefficient value for heme B pyridine hemochrome of 34.4 $mM^{-1}cm^{-1}$ at 557 nm (in the absolute spectrum) (Fuhrhop & Smith, 1975) was used. Protein concentration was determined by a modified Lowry method (Markwell et al., 1981) and by the Bradford method (Bradford, 1976). Bovine serum albumin was used as a standard in each method. Concentration of the standard solution was assessed spectrophotometrically using an extinction coefficient of 6.60 $\%^{-1}cm^{-1}$ at 280 nm. The heme content of the purified 101F6-His_8 protein was found to be 1.59 (±0.06) mole/mole protein, as calculated from the absolute spectrum at 557 nm of the pyridine hemochrome. This value was slightly less than the calculated value for bovine CG cytochrome b_{561} at 1.70 mole heme/mole protein (Tsubaki et al., 1997). Nevertheless, the value supported the presence of two heme B groups *per* protein in the purified sample, like the classic CG cytochrome b_{561}. This result was also consistent with the result of EPR analysis on the purified form of oxidized 101F6-His_8 (Recuenco et al., unpublished), which showed the presence of two independent heme centers.

Mass spectrometric analyses were conducted with a Voyager DE Pro mass spectrometer (Applied Biosystems, Foster City, California, USA). The estimated molecular mass of the intact 101F6-His_8 was found as 25941.70 Da, very close to the theoretical value corresponding to 1-240 residue (25996.9 Da; average). In total of 240 amino acid residues of

the 101F6-His_8 protein, we could identify most of the cleaved peptides with coverage of more than 99% and there was no post-translational modification occurred. These results confirmed the successful expression and purification of the intact 101F6-His_8 protein.

Purification steps	Total protein (mg)	Total cytochrome b_{561} content (nmol)	Specific content (nmol/mg protein)	Purification Fold	Yield (%)
Microsomes	165.75	861	5.20	1	100
β-octyl glucoside-solubilized	109.20	459	4.20	0.81	53.3
Ni-NTA chromatography/desal/ concentrate	0.60	176	293.3	56	20.5

Table 1. Purification of recombinant human 101F6-His_8 protein (starting from a culture of 250 mL-scale).

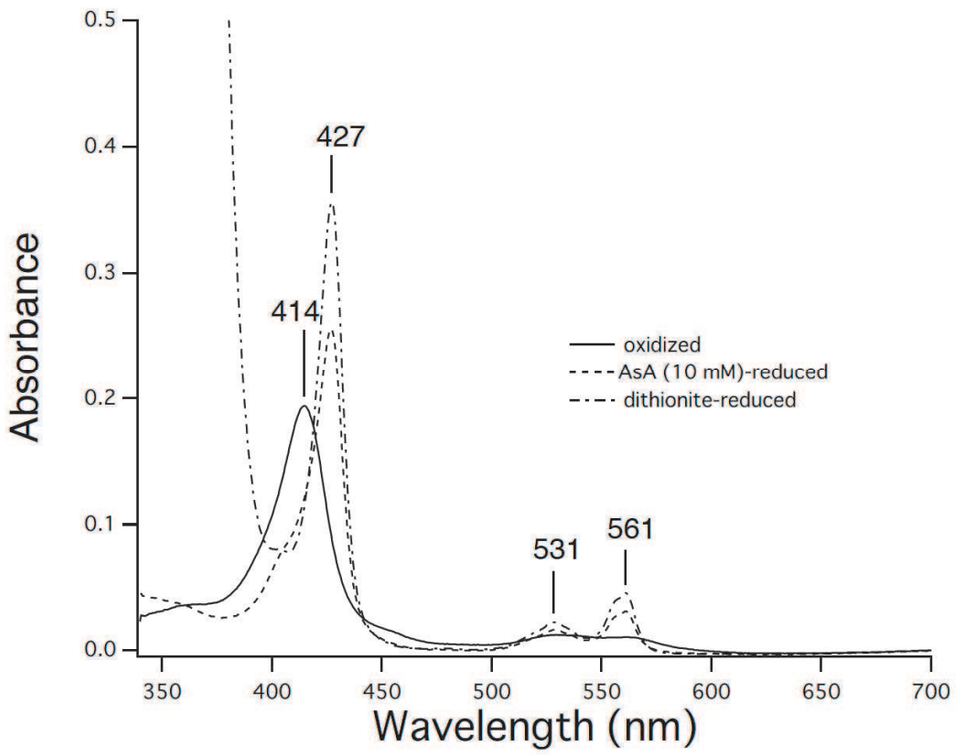


Fig. 1. Visible absorption spectra of purified 101F6-H_8 protein. Spectra were measured in oxidized (solid line), AsA (10 mM)–reduced (broken line), and dithionite-reduced (dashed broken line) states with cytochrome b_{561} content of 1.66 μM in 50 mM potassium phosphate buffer (pH 7.0) 1.0% β-octyl glucoside at room temperature.

The 101F6-His_8 protein was found to be reducible by AsA very efficiently. Redox titration analysis showed that its redox behaviour could be simulated satisfactory by assuming the presence of two independent heme *b* prosthetic groups with their midpoint potentials at +89.5 and +13.1 mV, respectively, slightly lower than the corresponding values of bovine CG cytochrome b_{561} (Tsubaki et al., 2000; Takeuchi et al., 2001). Electron accepting activity from AsA to the oxidized 101F6-His_8 protein and electron donating activity from the reduced 101F6-His_8 protein to MDA radical were further analyzed by a stopped-flow and pulse-radiolysis techniques, respectively. The results showed that these two properties are distinct from bovine CG cytochrome b_{561}: (a) very high electron accepting rate constant from ascorbate compared to other cytochrome b_{561} (Kobayashi et al., 1998); (b) absence of initial pH-dependency from AsA to the oxidized heme of 101F6-His_8 protein (Takigami et al., 2003). Details were described elsewhere (Recuenco et al., unpublished results). These properties may be directly related to the tumor suppressor activity of 101F6-His_8 protein in human lung tissues.

3.2 Protein crystallization screening

Purified eight-histidine-tagged human 101F6 protein in 50 mM potassium phosphate buffer (pH 7.0) containing 1% β-octyl glucoside was freed from aggregates by centrifugation at 15,000 rpm for 5 minutes. The supernatant containing the solubilized protein was put in Amicon Ultra-4 centrifugal filter unit (UFC 801096; Nihon Millipore K.K., Tokyo, Japan) and centrifuged at 3,000 rpm to concentrate the protein sample. The concentrated sample was again centrifuged to remove aggregates and then filtered through a 0.22 µm syringe filter. A 0.3 mL sample with a concentration at least 150 µM of cytochrome b_{561} content was enough for screening approximately 1000 different conditions using a crystallization robot, Hydra II Plus One system (Matrix Technologies Corp., Thermo Fischer Scientific Inc., Hudson, New Hampshire, USA). Screening of the crystallization condition was performed by a sitting-drop vapor diffusion method on 96-well Intelli-plates at room temperature. A ratio of 0.3 µL protein and 0.3 µL precipitant over 30 µL well solution was employed. Crystallization screening solutions such as, Classics Neo, Classics Neo II, JCSG+, Mb Class I, MPD, pH clear, pH clear II, $AmSO_4$, PEG I, and PEG II Suite (Qiagen K. K., Tokyo, Japan), were used. After the deposition of the samples, plates were incubated at 20°C for about 14 days.

Crystals with size of 0.020 mm were formed from the following precipitants:
JCSG+ (20 % PEG3350, 0.2 M sodium thiocyanate), Classics Neo (1.0 M imidazole (pH 7.0), and Classics II (25 % PEG3350, 0.2 M ammonium acetate, 0.1 M *bis*-Tris (pH 5.5) (Figure 2). Diffraction data were first measured on an R-axis imaging plate (IP) area detector (Rigaku Corp., Tokyo, Japan) at the Protein Design Laboratory, Yokohama City University. The X-ray diffraction data of the crystals were further collected at the Photon Factory in Tsukuba, Ibaraki, Japan (Beam line; PF-BL5A). The crystals from Classics II (25 % PEG3350 (w/v), 0.2 M ammonium acetate, 0.1 M *bis*-Tris (pH 5.5) were able to produce an X-ray diffraction pattern with a maximum resolution at 8.4 Å (Figure 2B). Further, optimization of the conditions using 20 % PEG3350 (w/v), 0.2M ammonium acetate, 0.1 M *bis*-Tris (pH 5.5) and 15 % PEG3350 (w/v), 0.2 M ammonium acetate, 0.1 M *bis*-Tris (pH 5.5) produced better crystals (Figure 3). The crystals from 15 % PEG3350 (w/v), 0.2M ammonium acetate, 0.1M *bis*-Tris (pH 5.5) produced a diffraction pattern of spots with the highest resolution at 4 Å (Figure not shown).

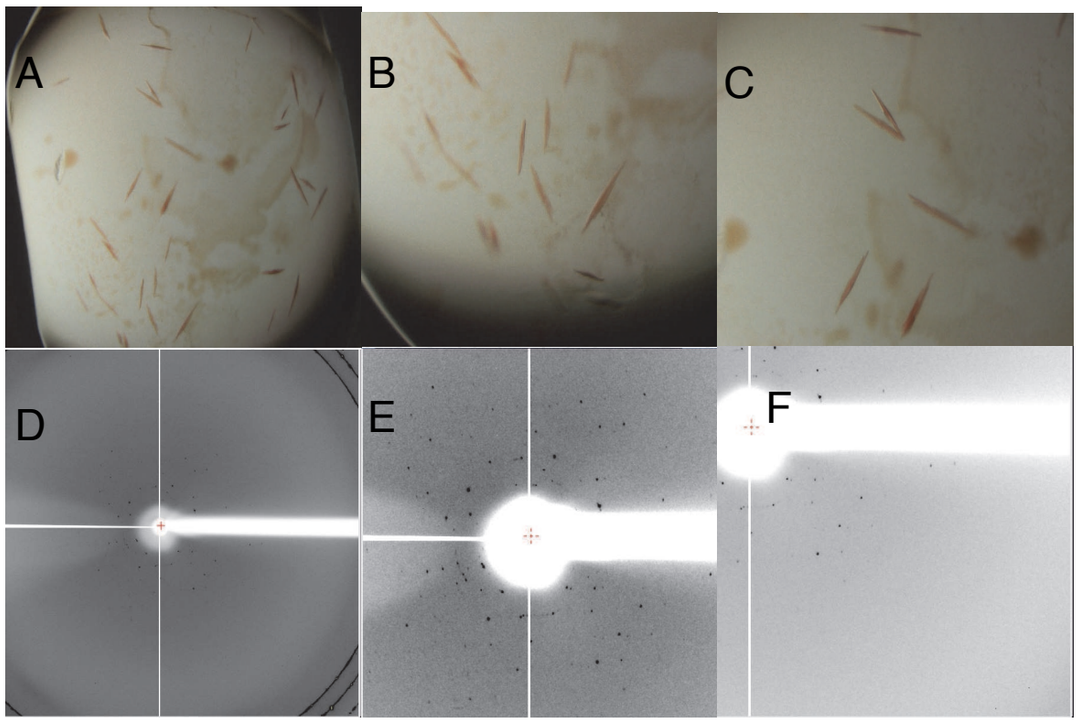


Fig. 2. Screening of crystallization conditions for 101F6-His_8 protein. (A-C) Crystals formed upon crystallization screening with a sitting-drop vapor diffusion method on 96-well plates. Conditions: Precipitant, 25% PEG3350, 0.2M Ammonium acetate, 0.1M *bis*-Tris (pH5.5) solution; Temperature 20 °C; Incubation time 10-14 days; Protein concentration, 150 μM in 50 mM potassium buffer (pH 7.0) containing 1.0 % β-octyl glucoside. (D-F) Diffraction patterns of the 101F6-H_8 crystals obtained from the conditions described above. Highest resolution at 8 Å was obtained at Tsukuba Photon Factory (Beam Line: PF-BL5A, Exposure time=20 sec).

4. Discussion

The candidate tumor suppressor protein 101F6 could be an important factor in cell proliferation and apoptosis. The reported induction of apoptosis in cultured cancer cells and inhibition of tumor growth in mouse models on forced expression of the 101F6 gene (Ji et al., 2002; Ohtani et al., 2007) gave a promising outlook for cancer prevention and treatment. In the present study, the human 101F6 protein was expressed and purified successfully from the yeast *Pichia pastoris* cells. The purified 101F6-His_8 protein was found to be reducible by AsA very efficiently and can donate electrons to MDA radical very rapidly. These new findings provide clues as to the possible role of the 101F6 protein in AsA-recycling that may be linked to processes that ultimately lead to apoptosis (Figure 3). Our view is basically consistent with the proposal by Ohtani et al., in which the exogenously-expressed 101F6 protein in cancer cells enhanced intracellular uptake of AsA, leading to an accumulation of cytotoxic H_2O_2 and synergistic killing of tumor cells through caspase-independent apoptotic and autophagic pathways (Ohtani et al., 2007). Our present view, however, needs further clarification to explain the possible mechanism(s) to enhance the cellular uptake of AsA by

the introduction of the 101F6 protein into the ER membranes. Further, the proximate target of the 101F6 protein in the proposed AsA-signaling pathway must be clarified. Importantly, in our scheme in Figure 3, H_2O_2 was not included. Ohtani et al. observed a significant increase in the intracellular accumulation of H_2O_2 in non-small cell lung cancer (NSCLC) cells only in response to exogenous 101F6 and AsA (Ohtani et al., 2007). Therefore, it might be very important to clarify the molecular mechanism concealed in the 101F6 protein in facilitating the formation and accumulation of cytotoxic H_2O_2 by the increased concentration of cytosolic AsA. For these purposes, complete three-dimensional structural information about this hydrophobic membrane protein is highly necessary and it would be very helpful for the understanding of the mechanism of the putative tumor suppression function and, further, in the design of therapeutic agents for cancer.

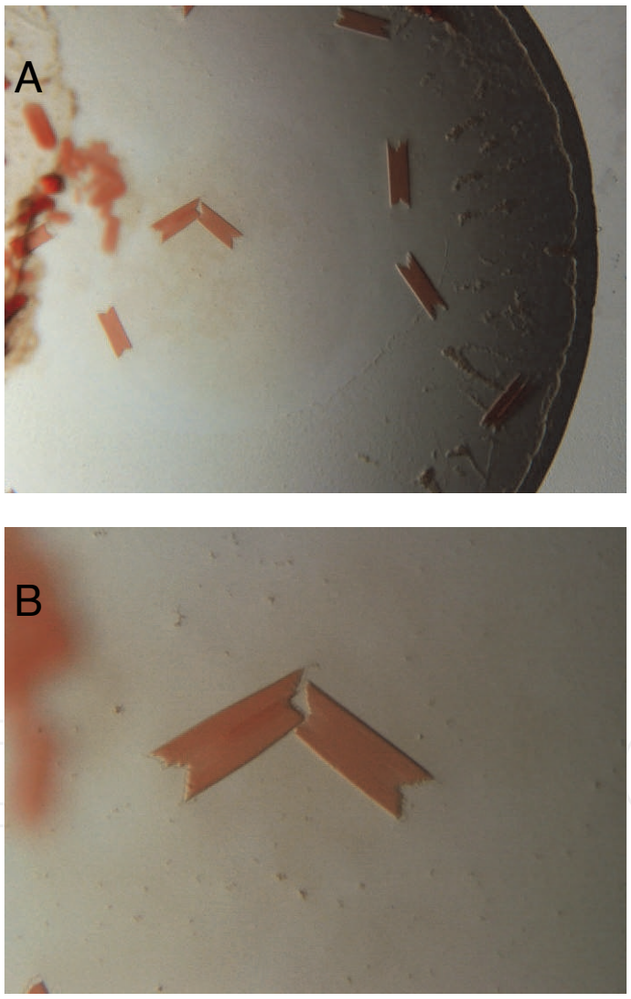


Fig. 3. Crystals formed from crystallization screening with a sitting-drop vapor diffusion method after optimization of precipitant composition. (A and B) 15% PEG3350 (w/v), 0.4M Ammonium acetate, 0.1M *bis*-Tris (pH6.0). Temperature 20°C; Incubation time, 10-14 days; Protein concentration, 150μM of 101F6-His_8 protein in 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 % β-octyl glucoside.

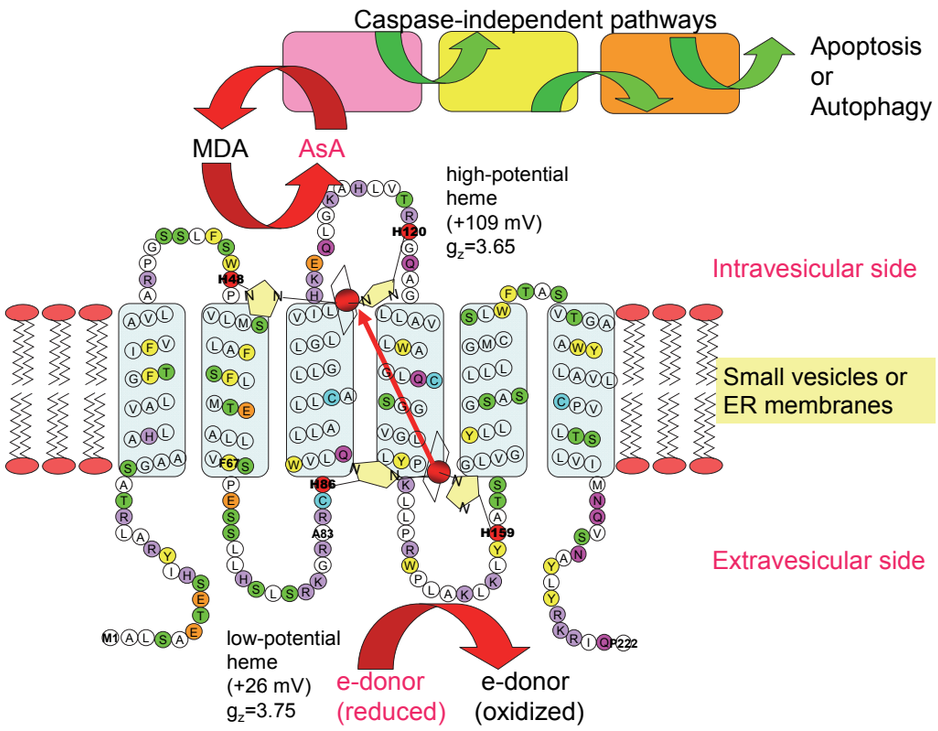


Fig. 4. Proposed topological model and function of the human tumor suppressor 101F6 protein in human cells. The human 101F6 protein contains six transmembrane α-helices and two *b*-type hemes. Alignment with other cytochromes b_{561} identified four histidine residues that might be the axial ligands of two hemes: His48 and His120 for the intravesicular heme; and His86 and His159 for the cytosolic side heme. The 101F6 protein may be located in the small vesicle or ER membranes. The 101F6 protein may function as an electron transfer protein inside the vesicles for AsA recycling. An electron donor donates an electron to the cytosolic heme of the 101F6 protein. The electron is then passed to the intravesicular heme *via* intramolecular electron transfer. MDA radical that might have been generated in some processes in the lumen accepts the electron from the intravesicular heme to re-generate AsA. AsA in the lumen of the vesicles is used as a cofactor to activate a certain target protein that may signal a caspase-independent pathway to induce apoptosis or autophagy.

Purification and crystallization of membrane proteins are considered to be much more difficult than soluble proteins. This difficulty is mostly due to the presence of transmembrane domains, in which hydrophobic α-helical domains were covered with detergent molecules, which was inevitably used for the solubilization of the proteins, forming a micelle structure. Such hydrophobic domains do not have specific interactions with other protein molecules, having a tendency to form non-specific aggregates during the protein purification, therefore, hampered the purification of membrane proteins into a homogenous state. Similar problems occur during the crystallization of a membrane protein. To obtain successful protein crystals of a membrane protein, we may have two strategies; one way is increasing a part of the hydrophilic domain to facilitate the specific interactions among the hydrophilic amino acid residues of the protein. Usually, a monoclonal body specific to such a hydrophilic domain would be used for such purpose. The other way is a choice of detergents. To find the best conditions for crystal growth with a suitable crystal group, one needs large amounts of pure protein sample and suitable detergents (Gutmann, 2007; Wetterholm et al., 2008).

We could obtain enough amounts of nearly homogeneous sample of human 101F6 protein in the form of eight-histidine-tagged fusion protein from the heterologous expression system of yeast *Pichia pastoris* cells. The protein samples were subjected to crystallization trials where initial screening was performed using a crystallization robot and commercially-available protein crystallization precipitants containing varying proportions of different salts, polymers and organic solvents. This allowed the screening of about 1000 different conditions within a very short time and usage of a tiny amount of the protein sample.
The β-octyl glucoside detergent used throughout in this study was a small micelle-forming detergent. Small micelle-forming detergents solubilize membrane proteins efficiently and leave hydrophilic parts of the protein being exposed to the water medium, allowing better interactions with other protein molecule(s) that are required for the formation of protein crystal lattice (Gutmann, 2007). However, if the hydrophobic regions are not well-covered with detergent molecules, aggregation of the membrane protein may occur. Large micelle-forming detergents such as n-dodecyl-β-D-maltoside and polyoxyethylene dodecyl ether can be used to address this problem. But with the use of these detergents, only small portions of the protein may be actually available for the lattice formation with other protein molecules. Thus, the human 101F6 protein should be subjected to a detergent screening to obtain better diffracting crystals and resolve its X-ray structure.

5. Conclusion

Previous studies showed that forced expression of the *101F6* gene in cultured cancer cells and in animal cancer models could significantly inhibit tumor growth. These promising results encouraged our investigation on the properties and function of the 101F6 protein. The human 101F6 protein was successfully expressed as an octahistidine-tagged fusion protein in the methylotropic yeast *Pichia pastoris* and was purified in its functional form. Characterization of the protein revealed that it possesses similar absorption spectra and AsA-reducibility as the prototype bovine CG cytochrome b_{561}. However, the results from kinetic studies on the reduction by AsA and the oxidation by MDA radical indicated different properties in the electron transfer mechanism. Most of the differences may be attributed to the low sequence homology of the 101F6 protein to the bovine CG cytochrome b_{561}. Other previously studied members of the cytochrome b_{561} family are much more similar to each other because of their higher sequence homology. Present study showed that it was possible to produce high quality sample and good diffracting crystals of the octahistidine-tagged 101F6 protein. An extensive screening for the best condition for crystallization should be done with the use of other detergents (such as n-dodecyl-β-D-maltoside), precipitants, and monoclonal antibodies. The detailed protein structure of the 101F6 protein through X-ray crystallography may be attained in the near future.

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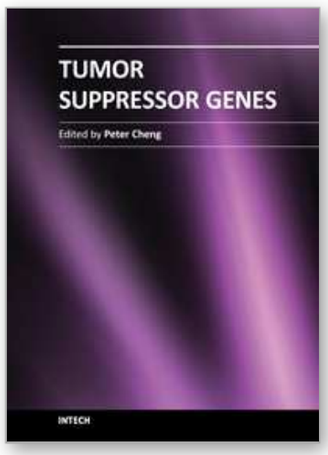
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Tumor Suppressor Genes
Edited by Dr. Yue Cheng

ISBN 978-953-307-879-3
Hard cover, 332 pages
Publisher InTech
Published online 03, February, 2012
Published in print edition February, 2012

Functional evidence obtained from somatic cell fusion studies indicated that a group of genes from normal cells might replace or correct a defective function of cancer cells. Tumorigenesis that could be initiated by two mutations was established by the analysis of hereditary retinoblastoma, which led to the eventual cloning of RB1 gene. The two-hit hypothesis helped isolate many tumor suppressor genes (TSG) since then. More recently, the roles of haploinsufficiency, epigenetic control, and gene dosage effects in some TSGs, such as P53, P16 and PTEN, have been studied extensively. It is now widely recognized that deregulation of growth control is one of the major hallmarks of cancer biological capabilities, and TSGs play critical roles in many cellular activities through signaling transduction networks. This book is an excellent review of current understanding of TSGs, and indicates that the accumulated TSG knowledge has opened a new frontier for cancer therapies.

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Mariam C. Recuenco, Suguru Watanabe, Fusako Takeuchi, Sam-Yong Park and Motonari Tsubaki (2012). Properties of Human Tumor Suppressor 101F6 Protein as a Cytochrome b561 and Its Preliminary Crystallization Trials, Tumor Suppressor Genes, Dr. Yue Cheng (Ed.), ISBN: 978-953-307-879-3, InTech, Available from: http://www.intechopen.com/books/tumor-suppressor-genes/properties-of-human-tumor-suppressor-101f6-protein-as-a-cytochrome-b561-and-its-preliminary-crystall

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