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Electron Transfer Reactions of Candidate Tumor Suppressor 101F6 Protein, a Cytochrome b_{561} Homologue, with Ascorbate and Monodehydroascorbate Radical

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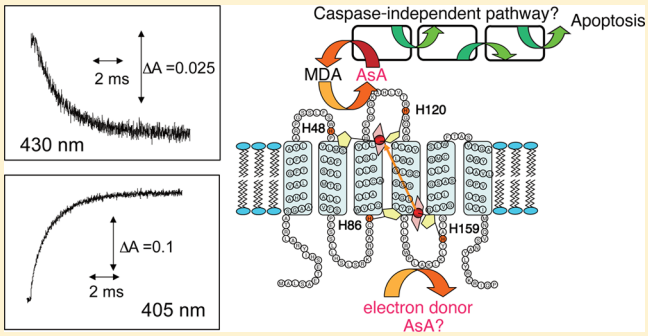
Supporting Information

ABSTRACT: The candidate tumor suppressor 101F6 protein is a homologue of adrenal chromaffin granule cytochrome b_{561}, which is involved in the electron transfer from cytosolic ascorbate to intravesicular monodehydroascorbate radical. Since the tumor suppressor activity of 101F6 was enhanced in the presence of ascorbate, it was suggested that 101F6 might utilize a similar transmembrane electron transfer reaction. Detailed kinetic analyses were conducted on the detergent-solubilized recombinant human 101F6 for its electron transfer reactions with ascorbate and monodehydroascorbate radical by stopped-flow and pulse radiolysis techniques. The reduction of oxidized 101F6 with ascorbate was found to be independent of pH in contrast to those observed for chromaffin granule and *Zea mays* cytochromes b_{561} in which both cytochromes exhibited very slow rates at pH 5.0 but faster at pH 6.0 and 7.0. The absence of the inhibition for the electron acceptance from ascorbate upon the treatment with diethyl pyrocarbonate suggested that 101F6 might not utilize a "concerted proton/electron transfer mechanism". The second-order rate constant for the electron donation from the ascorbate-reduced 101F6 to the pulse-generated monodehydroascorbate radical was found to be 5.0×10^7 M^{-1} s^{-1}, about 2-fold faster than that of bovine chromaffin granule cytochrome b_{561} and about five times faster than that of *Zea mays* cytochrome b_{561}, suggesting that human 101F6 is very effective for regenerating ascorbate from monodehydroascorbate radical in cells. Present observations suggest that 101F6 employs distinct electron transfer mechanisms on both sides of the membranes from those of other members of cytochrome b_{561} protein family.



It has been established that the adrenal chromaffin granule (CG) cytochrome b_{561} acts as an electron transfer protein across the membranes of adrenal chromaffin vesicles.[1,2] Ascorbate (AsA) inside the vesicles is used as a one-electron donor for dopamine β-hydroxylase that catalyzes the biosynthesis of adrenaline from dopamine. The monodehydroascorbate (MDA) radical generated during the process is, then, reduced back to AsA by accepting electrons from CG cytochrome b_{561}.[3−5] The oxidized CG cytochrome b_{561} would obtain reducing equivalents from cytosolic AsA via transmembrane electron transport. CG cytochrome b_{561} with an approximate molecular size of 28 kDa has a suitable structure for this function, containing six transmembrane α-helices and two heme *b* prosthetic groups on each side of the membranes.[6] Recently, it became apparent that various eukaryotic organisms bear genes coding for a putative membraneous protein, which has great similarities with a primary sequence of CG cytochrome b_{561}. Thus, a classification of a group of membraneous proteins called "cytochrome b_{561} protein family" was established.[7] Many following studies proved that the products of these eukaryotic genes were indeed members of the cytochrome b_{561} protein family. Well characterized homologues included duodenal cytochrome b_{561} (Dcytb),[8,9] Lcytb,[10] and *Zea mays* cytochrome b_{561},[11,12] all of which were shown to possess characteristic properties as a member of the cytochrome b_{561} family, such as the presence of two hemes *b*, similarities in absorption spectra, and the reducibility with AsA.

A 630-kb region in human chromosome 3p21.3 was identified as a critical region for lung cancer homozygous deletions.[13] Contiguous candidate tumor suppressor genes within this region had been identified. Six of these genes,

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namely, *101F6*, *NPRL2*, *BLU*, *RASSF1*, *FUS1*, *HYAL2*, and *HYAL1*, were used to construct recombinant adenovirus vectors for the transformation of the cancer cells and for animal model studies.[14] Upon forced expression of *FUS1*, *101F6*, and *NPRL2* genes in the cultured cancer cells, considerable inhibition of tumor growth by apoptosis and changes in cell cycle processes were observed.[14] Further, in experimental lung metastases mouse models, intratumoral injection of the protamine-complexed adenovirus vectors including Ad-*101F6*, Ad-*NPRL2*, Ad-*FUS1*, and Ad-*HYAL2* drastically controlled the tumor growth.[14] A more recent study showed that forced expression of 101F6 protein via nanoparticle-mediated gene transfer along with subpharmacological doses of AsA resulted in the significant suppression of the growth of lung cancer cells *in vitro*.[15] In contrast to normal lung cells, 101F6 protein would be lost in most of lung cancers.[15] In animal model studies, tumor growth in the *101F6*-nanoparticle and AsA-treated lung cancer mice was also significantly suppressed.[15]

These observations seemed to suggest that the *101F6* gene product is a genuine tumor suppression protein in human, although its molecular mechanism for the tumor suppression was scarcely known. Most importantly, this candidate human tumor suppressor gene *101F6* was predicted to code for a membrane protein that is homologous to cytochrome b_{561}.[7] This prediction was proved recently by employing heterologous expression systems.[16−20] The 101F6 proteins consist of a subfamily (E subfamily) very distant from other members of cytochrome b_{561} family, and, therefore, the putative AsA and MDA radical binding sequences are not well conserved[7] (Figure 1). However, since the purified 101F6 protein in oxidized form in detergent micelles was found to be reducible by AsA,[16,18,19] it might be inferred that AsA is also used as a physiological electron donor. Indeed, previous studies showed

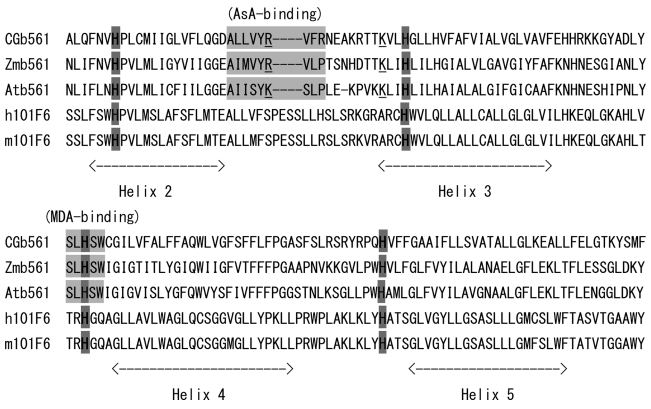

Figure 1. Comparison of amino acid sequences for the central domains of bovine adrenal CG cytochrome b_{561} (CGb561), *Zea mays* cytochrome b_{561} (Zmb561), *Arabidopsis thaliana* cytochrome b561 (Atb561), and human and mouse 101F6 proteins (h101F6 and m101F6). The central domain of the cytochrome b_{561} protein family contains four transmembrane helices (indicated by arrows). Within the domain, four heme-coordinating His residues (in dark gray background) are fully conserved, in which the first and third His residues coordinate intravesicular heme center, whereas the second and the fourth His residues bind to the cytosolic heme center. Putative AsA-binding and putative MDA-binding sequences are shown in gray background. Additionally, a Lys residue[11,26] and an Arg residue,[20,40] which were shown to have some important roles in AsA-binding, are underlined.

that the forced expression of the human *101F6* gene via adenovirus and nanoparticle gene transfer techniques increased the uptake of AsA and induced apoptosis in cultured cancer cells.[14,15] On the other hand, mouse 101F6 protein expressed in CHO cells was found to be localized in small vesicles, endosomes, and endoplasmic reticulum and may possess a transmembrane ferric reductase activity.[21] These observations suggest that a distinct mechanism is operative in 101F6 proteins different from those of CG or *Zea mays* cytochrome b_{561} for the electron transfer from AsA.

In this paper, we describe detailed kinetic studies on the electron transfer reactions of recombinant human 101F6 protein with AsA and MDA radical by stopped-flow and pulse radiolysis techniques. Present results provide valuable information to explain such unique properties of 101F6 protein and would be very useful to clarify its physiological activity in living cells as a putative tumor suppressor.

EXPERIMENTAL PROCEDURES

Expression and Purification of 101F6. The candidate human tumor suppressor protein 101F6 was expressed as an 8× histidine-tagged protein in *Pichia pastoris* GS-115 cells and was purified from microsomes by solubilization in buffer with β-octyl glucoside followed by affinity chromatography on a Ni-NTA Sepaharose column, as previously described.[18,20] The UV−visible absorption spectra and SDS-PAGE profiles were analyzed to check the purity of the sample. The concentration of 101F6 protein (101F6-H_8) was determined from the difference in absorbance at 561 and 575 nm using the extinction coefficient value of 27.7 mM^{-1} cm^{-1} employed for bovine CG cytochrome b_{561}.[11]

DEPC-Treatment of 101F6-H_8. Air-oxidized 101F6-H_8 solution was diluted to 6.5 μM with 50 mM potassium-phosphate buffer (pH 7.0) containing 1.0% (w/v) β-octyl glucoside. The sample was treated with 0.5 mM (final) of diethylpyrocarbonate (DEPC) for 60 min, as previously described.[12,20,22,23] During the DEPC-treatment, difference spectra in the UV−visible region were recorded every 5 min with a Shimadzu UV-2400PC spectrophotometer (Kyoto, Japan) at room temperature. The DEPC-treated sample was, then, gel-filtered through a PD-10 column equilibrated with 50 mM potassium-phosphate buffer (pH 7.0) containing 1.0% (w/v) β-octyl glucoside to remove unreacted DEPC. A control experiment was conducted by using methanol instead of the DEPC solution. The samples were concentrated and, then, examined for the electron accepting activity from AsA using a Shimadzu UV-2400PC spectrophotometer (Kyoto, Japan).[20] Both untreated and the DEPC-treated samples were, then, subjected to stopped-flow analyses.

Stopped-Flow Measurements. A stopped-flow spectrophotometer (RSP-100-03DR, UNISOKU Co. Ltd., Osaka, Japan) was used for rapid kinetic measurements on the reduction of oxidized 101F6-H_8 (or DEPC-treated 101F6-H_8) with AsA in β-octyl glucoside micelles.[24] The oxidized 101F6-H_8 (final, 2 μM) sample in 50 mM buffer of desired pH (sodium acetate buffer for pH 5.0, potassium phosphate buffer for pH 6.0 and pH 7.0) was introduced in one chamber of the apparatus. The other chamber contained AsA solution (2, 4, 8, 16 mM) in the same buffer. Temperature of the sample chambers and a sample mixing holder was maintained at 25 °C by connecting to a thermo-bath (Tokyo Rikakikai Co. Ltd., Model NCB-1200; Tokyo, Japan). The test solutions were mixed in a 1:1 (v/v) ratio. The heme reduction of 101F6−H_8

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was monitored spectrophotometrically by the changes in absorbance at 427 nm of the Soret band peak in various duration times (1, 10, and 60 s). Data points were collected in every 250 μs for the measurements of time duration of 1 s and in every 2.5 ms and 15 ms for the measurements of time duration of 10 and 60 s, respectively. The time-courses of the absorbance changes were fitted to a single exponential equation assuming a pseudo-first-order reaction using Igor Pro (v 6.03) software.

Pulse Radiolysis. Pulse radiolysis experiments were performed with an electronic linear accelerator at the Institute of Scientific and Industrial Research, Osaka University, as previously described.[25] The 101F6-H_8 sample in 50 mM potassium phosphate buffer pH 7.0 with 1% β-octyl glucoside and 10 mM AsA was placed inside of a quartz cell and then bubbled with N_2O gas for about 2 min. Under this condition, the lower potential heme (+26 mV) was almost fully reduced, whereas the higher potential heme (+109 mV) might be partially oxidized.[20] The concentration of the pulse-generated MDA radical was measured at 360 nm using a molar extinction coefficient of 3300 M^{-1} cm^{-1}. Oxidation and reduction of the 101F6−H_8 sample after the radiation pulse were monitored by following absorbance changes at 430 and 405 nm using a spectrophotometer.

RESULTS

Stopped-Flow Analysis for the Reaction with AsA. Stopped-flow kinetic measurements were performed to study the reduction of oxidized hemes of the recombinant human 101F6 protein by AsA. Figure S1, Supporting Information shows the traces for the changes in absorbance at 427 nm of 101F6-H_8 after mixing with AsA at different time scales and at different AsA concentrations. The increase in the absorbance at 427 nm corresponds to the reduction of the oxidized heme. In the initial reaction stages, the reduction of oxidized 101F6-H_8 with AsA was very quick irrespective of pH, although slightly slower at acidic pH. This observation was distinctly different from those reported previously for the reduction of oxidized bovine adrenal CG cytochrome b_{561}[26] and *Zea mays* cytochrome b_{561}[11] using stopped-flow technique. Both proteins had evident time-lags in the reduction process at pH 5.0 where the increase in absorbance at 427 nm in the first 1 s after the mixing was extremely slow.

For the kinetic analysis, each trace was fitted to a single exponential equation, in which the reaction was assumed to be pseudo-first-order as the AsA concentration in the final mixture would be in great excess over the concentration of 101F6 protein, and only one of the two hemes (i.e., the heme on the cytosolic side) was actually reacting with AsA. From the calculated apparent rate constants (Table S1), the highest apparent rate constants were obtained for the 1 s time domain for all the pH tested (pH 5.0, 6.0, 7.0) with values ranging from 3.5 to 6.7 s^{-1} (Table S1). There was a lowering of the apparent rate constant for the data from the longer time domain (10 and 60 s time domains). We chose the data from the 10 s time domain as the most appropriate ones for the detailed analysis. On the basis of the dependency on AsA concentration, the second-order rate constants were calculated and were found around 50 M^{-1} s^{-1} (pH 7.0, 52.5 M^{-1} s^{-1}; pH 6.0, 55.2 M^{-1} s^{-1}; pH 5.0, 45.0 M^{-1} s^{-1}) that were close to each other (Figure 2). (For the details on the kinetic analysis, see Appendix of the Supporting Information.)

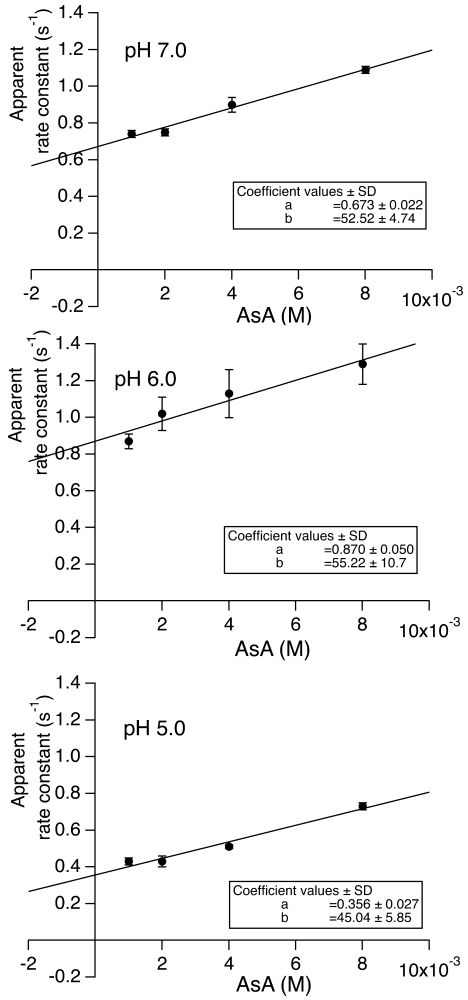

Figure 2. Determination of the second-order rate constant for the reaction of the oxidized form of human 101F6-H_8 with AsA at three different pHs. The rate constant was determined by the dependency of the apparent rate constants on the AsA concentrations for 10 s time domain measured at three different pHs (pH 5.0, 6.0, and 7.0) by assuming a linear relationship of $y = bx + a$, where y is apparent rate constant, x is the concentration of AsA (M), and a is the intercept. Other conditions were described in the main text or in the Supporting Information.

Effects of DEPC-treatment on the electron accepting ability from AsA were examined. In our recent preliminary study,[20] we found that the DEPC-treatment of the recombinant human 101F6-H_8 in the oxidized state caused *N*-carbethoxylation of His residues as large as nine residues/molecule based on the absorption change at 244 nm. However, very different from bovine CG and *Zea mays* cytochromes b_{561}, the treatment did not affect significantly its electron accepting ability from AsA, based on a mixing assay using a conventional UV−vis spectrophotometer.[20] Present stopped-flow analyses confirmed that the DEPC-treated 101F6-H_8 (with a similar extent in the *N*-carbethoxylation with that used for the mixing assay) was not much different from the untreated control sample in its apparent rate constant for the electron accepting reaction from AsA at pH 7.0 (Figure S2A,B). Indeed, in the second-order rate constants, there was a slight decrease for the DEPC-treated sample compared to the control sample (67.1 vs. 75.8 M^{-1} s^{-1}) for the data from 10 s time-domain (Figure 3) but a slight

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increase (28.0 vs. 31.7 M^{-1} s^{-1}) for the data from 60 s time-domain.

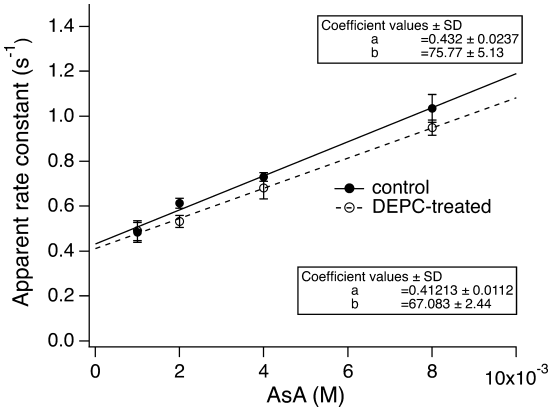

Figure 3. Effect of DEPC-treatment of recombinant human 101F6 on the heme reduction with AsA monitored by a stopped-flow method. Determination of the second-order rate constant based on the apparent rate constant of the heme reduction with AsA at different concentrations for 10 s time domain was conducted by assuming the linear relationship of $y = bx + a$, where y is apparent rate constant, x is the concentration of AsA (M), and a is the intercept. Other conditions were described in the main text or in the Supporting Information.

Pulse Radiolysis Analysis for the Reactions with MDA Radical and AsA. In a next step, we studied the ability of the AsA-reduced 101F6-H_8 protein to donate electron equivalents to MDA radical. Figure 4 shows absorbance changes of the reduced heme of 101F6 protein upon reaction with the radiation-pulse-generated MDA radical. At 430 nm, the rapid decrease in the absorbance indicated a decrease of the reduced heme level (Figure 4A, top). At 405 nm, on the other hand, the rapid increase in absorbance reflected the increase of the level of oxidized heme (Figure 4A, bottom). Since these two absorbance changes occurred very rapidly in a concerted manner, these fast phases were assigned to the electron donation from the reduced heme to MDA radical, as observed for bovine CG cytochrome b_{561}.[25] When a following slower reaction process was monitored at 430 nm in the 0.5−1 s time domain, an increase of the level of reduced heme was observed. This change was assigned as the re-reduction of oxidized heme of 101F6 protein with surrounding AsA in the medium (Figure 4B).

For the initial rapid process corresponding to the heme oxidation by MDA radical, we determined apparent rate constants at different concentrations of 101F6-H_8 at pH 7.0 (Figure S3) assuming a single exponential decay. Then, a second-order rate constant was calculated from the protein concentration dependency of the apparent rate constants. It was calculated as 5.0×10^7 M^{-1} s^{-1} (Figure 4C). This value was about twice the reported value for bovine adrenal CG cytochrome b_{561} (2.6×10^7 M^{-1} s^{-1})[25] and was much faster than the value for *Zea mays* cytochrome b_{561} (1.0×10^7 M^{-1} s^{-1}).[11] On the other hand, for the following slow process corresponding to the re-reduction of oxidized heme by AsA, a second-order rate constant was calculated similarly based on the protein concentration dependency of the apparent rate constants. It was found as 24×10 M^{-1} s^{-1} at pH 7.0, similar to that for *Zea mays* cytochrome b_{561} (25×10 M^{-1} s^{-1}) (Rahman et al., unpublished observation) and slightly lower than bovine adrenal CG cytochrome b_{561} (80×10 M^{-1} s^{-1}).[25]

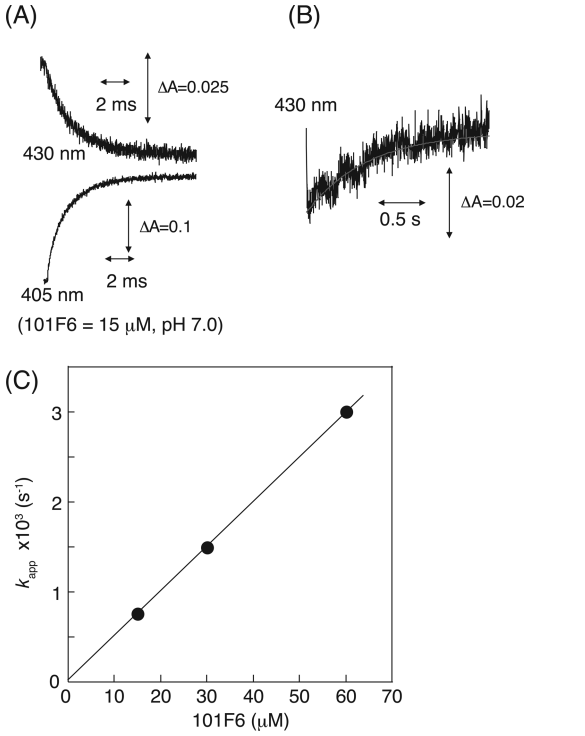

Figure 4. Oxidation of the AsA-reduced form of human 101F6-H_8 by radiolytically generated MDA radical and subsequent re-reduction of the heme with AsA. (A) Rapid oxidation of the reduced heme of human 101F6-H_8 was observed within 4 ms after the radiation pulse at pH 7.0. The upper panel shows the absorbance change at 430 nm near the Soret band peak at 427 nm of the reduced form of 101F6-H_8. The lower panel shows the change in absorbance at 405 nm, near the Soret band peak at 417 nm of the oxidized form. (B) Absorbance change at 430 nm of 101F6-H_8 in the time duration of 2 s after the radiation pulse at pH 7.0. Initial rapid decrease corresponding to the oxidation of the reduced heme was followed by a slower re-reduction of the oxidized heme by surrounding AsA. (C) Determination of the second-order rate constant based on the dependency of the apparent rate constants of the heme oxidation with MDA radical on the concentration of 101F6-H_8 protein.

Effects of changing pH on the fast electron donation reaction to MDA radical and the following re-reduction process of the oxidized heme with AsA were analyzed (Figure 5). Surprisingly, the fast electron donation reaction to MDA radical showed its optimum pH around 6−7 (Figure 5A), different from that of bovine adrenal CG cytochrome b_{561}. Bovine adrenal CG cytochrome b_{561} exhibited its optimum pH at 5.5 for the electron donation to MDA radical,[25] probably reflecting its preference for acidic conditions of the lumen of chromaffin vesicles. On the other hand, the slower re-reduction process of the oxidized heme with AsA in the medium showed its optimum pH around 6.5 for both 101F6 (Figure 5B) and bovine adrenal CG cytochrome b_{561}.[25]

DISCUSSION

Distinct Heme Environments of 101F6 Protein. The candidate human tumor suppressor protein 101F6 is considered to be a member of cytochrome b_{561} protein family, as described in our previous reports.[7,18,20] Like the prototype adrenal CG cytochrome b_{561}, it was a very hydrophobic heme-containing membrane protein and was reducible with AsA.[18] While the absorption spectral profile of 101F6-H_8 was

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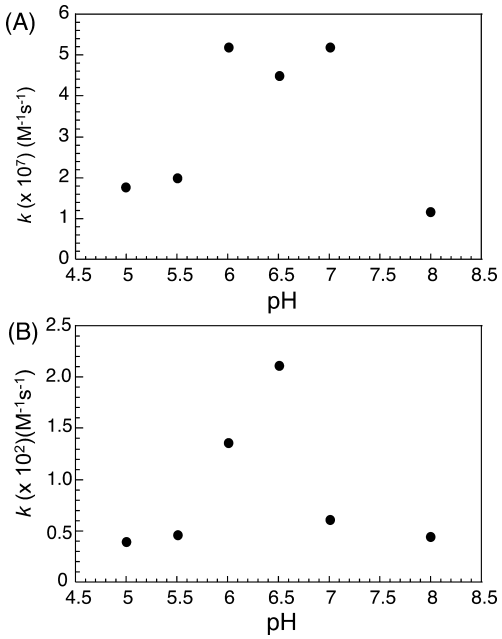

Figure 5. pH-dependencies of the rate constants for the reaction of MDA radical with AsA-reduced form of human 101F6-H_8 (A) and for the subsequent re-reduction of the oxidized heme with AsA (B). Experimental conditions are the same as in Figure 4. Other conditions are described in the text.

characteristic of cytochrome b_{561} proteins, the 101F6-H_8 protein had lower midpoint potentials (+109 mV and +26 mV)[18,20] than those of adrenal CG cytochrome b_{561} (+155 mV and +62 mV).[22] The EPR spectra of human 101F6-H_8 protein were also different from the usual spectral pattern of cytochrome b_{561}. For the adrenal CG cytochrome b_{561}, two EPR signals at g_z = 3.7 and g_z = 3.14 indicated the presence of two low-spin ferric hemes. The former g_z = 3.7 signal was assigned to a highly anisotropic (HALS) species, while the g_z = 3.14 signal was assigned to a rhombic heme species with a cytochrome b_5-like heme environment.[6] On the other hand, human 101F6-H_8 protein showed two overlapped HALS-type EPR signals at g_z = 3.75 and g_z = 3.65.[20] Recombinant mouse 101F6 protein, on the other hand, showed EPR spectra having signals at g_z = 3.61, g_z = 2.96, and g_y = 2.26 at 5, 10, and 15 K.[17] In this case, the former g_z = 3.61 signal seemed to correspond to the g_z = 3.65 HALS species.[20] However, other two EPR signals derived from a rhombic heme species were not observed. Thus, at least, one of the two heme centers seemed to have very different environments between human and mouse 101F6 proteins, and, more importantly, this heme center is unique among the heme centers of classical members of cytochrome b_{561} protein family. Since there were two HALS-type EPR signals being overlapped in the spectra of human 101F6 protein, we could not assign precisely the heme signals, but the g_z = 3.65 HALS species was more likely corresponding to the intravesicular heme and vice versa.[20]

We can attribute the unique biophysical properties of human 101F6 protein (i.e., split α-band peak, lowered redox potentials, and unique EPR spectra) to its lowered sequence similarity with those of other CG and *Zea mays* cytochromes b_{561} (Figure 1).[20] A closer examination of the sequences showed that the putative "MDA-radical binding motif" was not conserved and the "AsA -binding motif" were significantly modified ("modified motif 1") in the sequence of 101F6 proteins.[7] To examine whether such differences could influence its electron accepting ability from AsA, we first conducted stopped-flow experiments.

Distinct Electron Transfer Mechanisms of 101F6 from AsA. The reduction of oxidized heme of 101F6-H_8 by AsA seemed to be independent of pH of the medium based on the stopped-flow kinetic experiments (Figure S1; Figure 2). This was in a great contrast to those observed for bovine adrenal CG cytochrome b_{561} and *Zea mays* cytochrome b_{561}, in which both showed significant initial time-lags during the reduction process at pH 5.0.[11,26] For the explanation of the unique pH-dependency of the reduction with AsA, we assumed that a pH-dependent intermediate or a conformational change occurred at the putative AsA-binding site of cytochrome b_{561} protein.[26] A well-conserved lysine residue (Lys85 of bovine adrenal CG cytochrome b_{561} and Lys83 of *Zea mays* cytochrome b_{561}) within the putative AsA-binding site and one of the heme axial His ligands on the cytosolic side (His88 of bovine adrenal CG cytochrome b_{561} and His86 of *Zea mays* cytochrome b_{561}) of these proteins were found to have important roles for the quick electron acceptance from cytosolic AsA at neutral and basic pH.[11,27] In the usual cycle of electron acceptance from AsA, this conserved Lys residue has a guiding role for the access of a negatively charged AsA molecule to the binding site. Additionally, this Lys residue may accept a proton from the imidazole group of the heme axial His residue in the later stage of the course of the "concerted proton/electron transfer mechanism"[27] and, therefore, plays a part of the proton pathway.[11] Accordingly, the bound proton on the Lys residue has to be transferred quickly and appropriately to the outside of the protein moiety to begin a next cycle of the electron transfer reaction, and, therefore, this step is strongly pH-dependent. However, both human and mouse 101F6 proteins have an Ala residue at this position instead (Figure 1). Although there is a well-conserved Lys residue (only among the E subfamily[7]) located three residues "upstream" of the His ligand on 101F6 helix 5 (Figure 1), it is not clear whether this may have a compensating role for the missing Lys83 since it may be located more likely on the other side of the heme plane. Ala residues have a neutral side chain incapable of any protonation/deprotonation processes and could not be a part of the proton pathway nor a part of the interacting residues with a negatively charged AsA molecule. This view is consistent with the present results, i.e., absence of the pH-dependency upon the electron acceptance from AsA for human 101F6 protein.

The striking inhibitory effect of the DEPC-treatment on the electron accepting ability from AsA is one important aspect of the "concerted proton/electron transfer mechanism" operating in the cytochrome b_{561} protein family.[12,26−30] Since DEPC reagent specifically attacks a deprotonated nitrogen atom[31] (most likely $N_{\delta 1}$) of a heme-coordinating imidazole group of cytochrome b_{561}, which is the basis of the specific ability of this site for the concerted proton/electron transfer from AsA, almost complete absence of such an inhibitory effect of the DEPC-treatment on human 101F6 protein (Figure S2; Figure 3) would suggest that the "concerted proton/electron transfer mechanism" is not operating at the cytosolic heme center of human 101F6 protein. In other words, the $N_{\delta 1}$-carbethoxylation of axial His residue(s) does not occur for human 101F6 protein. Being consistent with this view, the DEPC-treatment of 101F6 protein did not affect the redox potentials of both heme centers significantly (10−15 mV of positive shift).[20] It must be emphasized again that, in addition to the replacement

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of the Lys residue, there are significant differences in the sequences around the putative AsA-binding site (Figure 1), and these differences are apparently responsible for the formation of specific characters of 101F6 protein upon the electron acceptance from cytosolic AsA.

The most straightforward way to evaluate the validity of our proposal would be an examination on the inhibition of the AsA-dependent heme reduction by the DEPC-treatment on the site-specific mutants. Recently, we have succeeded in preparing singly replaced mutants (F67Y, S68R, A83K) and a triply replaced mutant (F67Y/S68R/A83K) of 101F6 protein by mimicking those of adrenal CG or *Zea mays* cytochromes b_{561}. Surprisingly, the triply replaced mutant showed a significant inhibition of the AsA-dependent reduction after the DEPC-treatment. However, the DEPC treatment of the singly replaced mutants did show a quick heme reduction with AsA as observed for the wild-type 101F6 protein (Recuenco et al., personal communication). Therefore, we may conclude that if the putative AsA binding site was successfully restored, the specific $N_{\delta 1}$-carbethoxylation of heme-ligating imidazole could occur, and, accordingly, the AsA-dependent heme reduction was inhibited. Therefore, the lack of the inhibition by DEPC-treatment cannot be ascribed to a single amino acid residue but is rather ascribable to the entire organization of concerning amino acid residues around the putative AsA-binding site. We have extended the analysis further to examine whether the pH dependency of the AsA-dependent reduction could be restored as well for the triply replaced mutant (F67Y/S68R/A83K). However, there was no significant retardation of the fast electron transfer from AsA at an acidic pH (Recuenco et al., personal communication). The cause of the discrepancy is not clear at this moment; however, the pH dependency of the electron transfer reaction from AsA required a series of well-organized structural changes and proton transfers among AsA, heme, and surrounding amino acid residues. On the other hand, the specific $N_{\delta 1}$-carbethoxylation of heme axial imidazole required a deprotonated state of the imidazole group, which may be more easily attained by the replacement of three amino acid residues.

Distinct Electron Transfer Mechanisms of 101F6 Protein to MDA Radical. In considering the proposed physiological role of the intravesicular heme of the cytochrome b_{561} family for the electron donation to MDA radical, the reaction with MDA radical might occur at the intravesicular heme center of 101F6 protein as well. In our present pulse radiolysis experiments, AsA-reduced 101F6-H_8 was able to donate an electron equivalent to the pulse generated MDA radical very rapidly (Figure 4A). The calculated second-order rate constant was much faster than those of bovine adrenal CG cytochrome b_{561} (2-fold) and *Zea mays* cytochrome b_{561} (5-fold). Bovine adrenal CG cytochrome b_{561} exhibited its optimum pH at 5.5 for the electron donation to MDA radical,[25] whereas human 101F6 showed its optimum pH in neutral region (Figure 5A). This suggested that the molecular mechanism of the electron donation to MDA radical might be also different from other members of the cytochrome b_{561} family. Indeed, conservation of the amino acid sequence around the putative MDA radical binding site was also very different from those of classic-type cytochromes b_{561} (Figure 1). Further, the EPR spectral analysis of the human 101F6 protein in the oxidized state indicated that the high potential (+109 mV) heme center, which was accordingly assigned to the intravesicular heme and was attributed to be responsible for the electron donation to MDA radical, was converted to a highly anisotropic low-spin (HALS) species from an ordinary rhombic low-spin species of other cytochromes b_{561}.[20] Since this conversion is likely to be associated with drastic changes in the orientation of two imidazole planes of the heme axial His residues from parallel to perpendicular,[32,33] the putative architecture of the MDA radical binding site would be altered accordingly.

Rereduction of 101F6 Protein with a Bound AsA/Intramolecular Electron Transfer Reaction. One may notice that the second-order rate constants for the reaction with AsA obtained for the re-reduction phase of MDA radical-oxidized form of 101F6 showed significant pH-dependency (Figure 5B), which was very similar to the corresponding re-reduction process of bovine adrenal CG cytochrome b_{561}.[25] Further, the second-order rate constant value at pH 6.5 was probably about 3−4 times faster than those of corresponding values for the reaction of oxidized 101F6 protein with AsA obtained by the stopped-flow technique (Figure 2), which did not show any significant pH-dependency, as described in the previous section. These discrepancies are apparently due to the difference in the starting point of the electron transfer reaction. For the re-reduction phase of the MDA radical-oxidized form of 101F6 protein, the putative AsA-binding site might be preoccupied with an AsA molecule, being ready for supplying an electron to the oxidized heme on the extravesicular side. On the other hand, in the stopped-flow experiments, the entire heme reduction processes might be controlled by several factors: approaching of AsA to the catalytic site of 101F6 protein, binding of AsA at the catalytic site, electron transfer from AsA to the oxidized heme with a lower redox potential (i.e., extravesicular heme) by an unknown mechanism, leading to the intramolecular transfer reaction to the oxidized heme with a higher redox potential (i.e., intravesicular heme).[25] These factors are probably responsible for the offset of the linear relationship in the plots with large intercept values (Figure 2). It should be noted that the re-reduction of the MDA radical-oxidized heme is rate-limited by the step of intramolecular electron transfer from the reduced heme center on the extravesicular side to the oxidized heme center on the intravesicular side.[25] Therefore, it is very likely that the fundamental molecular mechanism controlling the intramolecular electron transfer reactions in the cytochrome b_{561} protein family might be very similar to each other, since the re-reduction phases for bovine adrenal cytochrome b_{561} and human 101F6 showed similar pH dependency, and, interestingly, their rate constants were not significantly different from each other. The distance between the two hemes centers would be higher than 2 nm based on a model calculation,[34] which far exceeded the maximum distance allowed for a long-range electron transfer by a single-step tunneling mechanism.[35] Thus, a hopping mechanism with coupled tunneling reactions would be a favorable choice [35] to explain a rather slow process from one heme to the other.

Putative Physiological Roles of 101F6 Protein and AsA for Tumor Suppression. In relation to the proposed function of 101F6 protein as a putative tumor suppressor,[14,15] it was reported that cancer cells treated with the nanoparticle-mediated 101F6 gene transfer had increased uptake of AsA and increased production of hydrogen peroxide, which might cause the induction of caspase-independent apoptosis and autophagy.[15] These findings provided a new insight into the molecular mechanism of tumor suppression by 101F6 protein and AsA.

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101F6 protein seems to be ubiquitously expressed in animal tissues, but in higher levels in the liver, kidney, and lung.[21] Indeed, it was reported that when mouse 101F6 protein was expressed in CHO cells it localized in small vesicles, including endosomes and endoplasmic reticulum of the perinuclear region[21] and may possess a ferric reductase activity when expressed in HEK 293T cells.[21] In our recent study using cultured A549 cells (originating from a human lung cancer tissue, lacking the expression of 101F6 protein), transfection of pcDNA3.1-h101F6 (containing the entire coding region of human *101F6* gene) showed a significant lowering of their cell proliferation and viability in the presence of AsA upon MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide)-based assays (Asada et al., personal communication). Further, simultaneous addition of menadione or tertiary butylhydroquinone in the medium enhanced the lowering of cell proliferation and viability (Asada et al., personal communication), suggesting that human 101F6 protein might participate in AsA-linked redox reactions in the ER membranes. On the basis of these observations and similarities to other members of the cytochrome b_{561} protein family, it is very likely that 101F6 proteins may be directly involved in AsA recycling, donating electrons to MDA radical in a fast and efficient manner. Indeed, our present study showed a very fast electron transfer reaction rate of the recombinant human 101F6 protein with MDA radical in neutral pH and proved that 101F6 protein is very effective for the reduction of MDA free radical to regenerate AsA (Figure 6).

Since the pulse radiolysis experiment indicated that the rate-limiting step of the whole trans-membrane reaction (AsA to 101F6 to MDA radical) is the intramolecular electron transfer between the hemes, the fast reaction between the reduced 101F6 and MDA radical would be irrelevant to the supply of the electron equivalent from the cytosolic heme center. Therefore, 101F6 probably would use only the electrons residing on the intravesicular side under the usual physiological condition. Such quick removal of the MDA radical from the intravesicular side might be sufficient to promote the hypothetical signaling pathway to cause caspase-independent apoptosis and autophagy. Since caspase-independent apoptosis and autophagy involved a pathway(s) starting from endosomes and endoplasmic reticulum, it is likely that AsA in the lumen of these organelles is directly used as a signaling messenger via a redox-signaling pathway. As an alternative possibility, such AsA in the lumen might be directly used for the production of reactive oxygen species (ROS) to initiate the apoptosis or autophagy. Indeed, Ohtani et al. reported that significant enhancement of intracellular H_2O_2 accumulation occurred upon expression of 101F6 protein and incubation with AsA.[15]

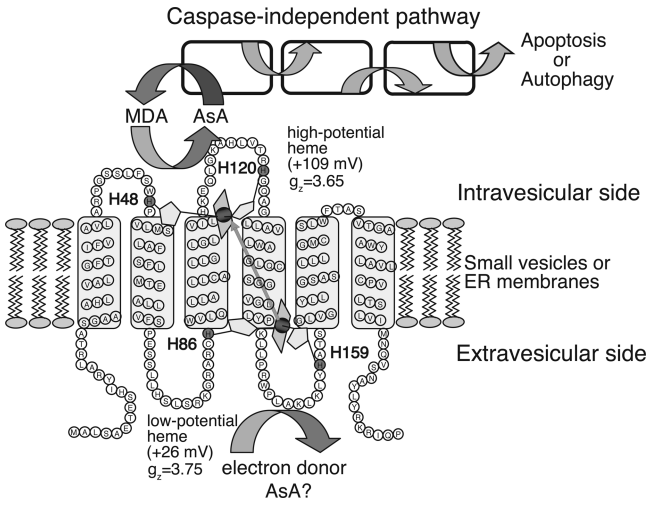

Figure 6. Proposed topological model and function of the human tumor suppressor 101F6 protein in human cells. Human 101F6 protein contains six transmembrane α-helices and two *b*-type heme prosthetic groups. Four conserved His residues are the axial ligands for the two hemes: His48 and His120 for the intravesicular heme; and His86 and His159 for the cytosolic heme. Human 101F6 protein may be located in the small vesicle or ER membranes. A cytosolic electron donor donates an electron to the cytosolic heme of 101F6 protein. The electron is then passed to the intravesicular heme via an intramolecular electron transfer. MDA radical that might have been generated from some processes in the vesicles accepts the electron from the intravesicular heme to regenerate AsA. AsA in the lumen of the vesicles is used as a cofactor to activate a certain protein that may signal a caspase-independent pathway to induce apoptosis or autophagy.

On the other hand, a physiological electron donor to the cytosolic heme center of the 101F6 protein is still obscure (Figure 6). AsA on the cytosolic side of the cell is apparently the most likely candidate. Quercetin[36] and other water-soluble flavonoids might be alternative candidates for the physiological electron donor to donate electrons to cytosolic heme center. However, in considering such physiological electron donor to 101F6 protein, we may have an alternative possibility that 101F6 might be able to receive electrons from some other electron donor(s) in the membranes, directly to the intravesicular heme center in addition to AsA or water-soluble electron donor(s). In this case, the rate-limiting step of the whole electron transfer is not the intramolecular electron transfer between two hemes. Indeed, such a possibility was raised previously[7] based on the slightly different putative electron receiving sequence (modified motif 1) and very different putative electron donating sequence (motif 2) among the members of group E subfamily. Recent studies by Berczi et al. suggested that dihydrolipoic acid (DHLA) could be a good electron donor for various cytochromes b_{561} including 101F6.[37,38] If these kinds of lipophilic electron donors, like CoQ_{10}, tocopherols, and dihydrolipoic acid, were actually functioning as a physiological electron donor, a completely different view for the role of 101F6 protein would emerge.

In conclusion, we argued previously that the "the concerted proton/electron transfer mechanism" upon electron acceptance from AsA[27] is maintained by only a limited number of conserved residues on the cytosolic side of cytochrome b_{561} protein family, which include two heme axial His residues (His86 and His159), Lys83, Arg72, and Tyr71 (with the numbering of *Zea mays* cytochrome b_{561}),[39] and these residues are also responsible for the specific DEPC reactivity.[12] Apparently, such a molecular mechanism does not exist in 101F6 proteins, suggesting that 101F6 protein may work under environments where there is no need to consider about the back-flow of the electron equivalents through it.[39] To verify our present proposal, however, a detailed structural study based on the X-ray crystallographic data might be highly necessary.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Three supplemental figures (Figures S1, S2, and S3), a supplemental table (Table S1), and an appendix describing "Transient kinetic analysis of the reduction of 101F6 with AsA

by a stopped-flow method". This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS USED

CG, chromaffin granule; AsA, ascorbate; MDA, monodehydroascorbate; HALS, highly anisotropic low-spin; DEPC, diethylpyrocarbonate; EPR, electron paramagnetic resonance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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