The membrane topological analysis of 3 β -hydroxysteroid- Δ 24 reductase (DHCR24) on endoplasmic reticulum

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Abstract

DHCR24 encodes 3 β -hydroxysteroid- Δ 24 reductase, catalyzing the conversion of desmosterol to cholesterol. Our previous study demonstrated that DHCR24 exerts an anti-apoptotic function as a reactive oxygen species (ROS) scavenger, for which it needs its FAD-binding domain. The membrane topology of DHCR24 on endoplasmic reticulum (ER) and the functional significance of its FAD-binding domain are not completely understood. Based on the structure predicted by bioinformatics, we studied the membrane topology of DHCR24 in murine neuroblastoma cells (N2A), using the fluorescent protease protection (FPP) technique. We showed that full-length DHCR24 is localized to the membrane of ER, whereas the predicted transmembrane (TM) domain-deleted DHCR24 mutation is localized to the cytoplasm. The change of DHCR24 localization suggests that the N-terminal TM domain is essential for the ER membrane targeting of DHCR24. The FPP assay demonstrated the membrane topology of DHCR24 with an N-terminal luminal/C-terminal cytoplasmic orientation. Measurement of intracellular ROS using H₂DCFDA revealed that the ROS levels of cells infected by plasmids driving expression of full-length DHCR24 or the TM domain-deleted DHCR24 mutation after H₂O₂ exposure were lower than those of control cells, suggesting that the ER membrane targeting of DHCR24 is not required for its enzymatic ROS scavenging activity. Confocal fluorescence microscopy revealed that the DHCR24-overexpressed cells were protected from apoptosis in response to oxidative stress, which was accompanied by a decrease in DHCR24 content on the ER and activation of caspase-3, suggesting that the anti-apoptotic function of DHCR24 is associated with its cleavage by caspase.

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Introduction

DHCR24 encodes 3 β -hydroxysteroid- Δ 24 reductase, which catalyzes the synthesis of cholesterol from desmosterol and belongs to a family of FAD-dependent oxidoreductases (Waterham *et al.* 2001). *DHCR24* mRNA comprises an ORF of 516 amino acid with a calculated molecular weight of 60.1 kDa, with a potential N-terminal secretory signal sequence, and with at least one putative transmembrane (TM) helix (Mushegian & Koonin 1995). In recent years, it has been demonstrated that DHCR24 is an endoplasmic reticulum (ER)-resident, multifunctional enzyme that possesses anti-apoptotic and cholesterol-synthesizing activities.

The *DHCR24* gene is expressed in all types of cells tested and loss-of-function gene mutations cause a cholesterol biosynthesis disorder, desmosterolosis (Waterham *et al.* 2001). In addition to its cholesterol-synthesizing activity, several biologically important activities of DHCR24 have been reported to date. DHCR24 interacts with p53 and increases its stability,

thereby regulating cell growth, senescence, and apoptosis (Wu *et al.* 2004, Kuehnle *et al.* 2008). Furthermore, using patient specimens, others and we have reported that decreased *DHCR24* expression is associated with apoptosis and its expression is required for cell survival (Sarkar *et al.* 2001, Di Stasi *et al.* 2005) and decreased *DHCR24* expression has also been implicated in neuronal cell death in brain regions affected by Alzheimer's disease (Greeve *et al.* 2000, Benvenuti *et al.* 2005). Conversely, overexpression of DHCR24 can protect the cell from apoptosis induced by oxidative stress (Greeve *et al.* 2000, Di Stasi *et al.* 2005, Lu *et al.* 2008). These studies suggest that DHCR24 possesses anti-apoptotic activity in many cell types. We have also observed that DHCR24 can protect cells from ER stress-induced apoptosis by scavenging excess reactive oxygen species (ROS; Lu *et al.* 2009). The mechanisms of the anti-apoptotic function of DHCR24 have been studied by several groups.

It was first reported that DHCR24 effectively inhibits the activation of caspase-3, a key mediator of the

apoptotic process (Greeve *et al.* 2000). In this case, DHCR24, by inhibiting the apoptotic cascade, was associated with a more aggressive behavior of tumor cells and with resistance to pharmacological treatment. Recent biochemical and clinical studies unveiled that DHCR24 is implicated in neuroprotective processes by modulating the level of cholesterol in membrane, which is associated with amyloid precursor protein (APP) processing and amyloid β (A β) generation *in vivo* (Cramer *et al.* 2006, Cecchi *et al.* 2008). In contrast, it was also reported that reduced *DHCR24* expression results in enhanced Golgi-localized γ -ear-containing ARF-binding protein 3 (GGA3) depletion, which further leads to augmented post-translational stabilization of β -site APP-cleaving enzyme 1 (BACE1) and increased β -amyloidogenic processing of APP (Lu *et al.* 2008, Sarajarvi *et al.* 2009). We previously demonstrated that the ability of DHCR24 to protect mouse embryonic fibroblasts and PC12 (rat adrenal pheochromocytoma cell line) cells against apoptosis elicited by oxidative stress is due, at least in part, to the scavenger activity of this protein (Lu *et al.* 2008). This result again provides evidence for an anti-apoptotic function of DHCR24 that is independent of APP and A β . An *in vitro* study using DHCR24 and its mutants demonstrated that the FAD-binding domain is necessary for its H₂O₂ scavenging function (Lu *et al.* 2008).

Several reports have shown that DHCR24 is mainly located on ER (Greeve *et al.* 2000, Lu *et al.* 2008, Battista *et al.* 2009). This is in accordance with most enzymes involved in *de novo* cholesterol biosynthesis, such as HMG CoA reductase, the rate-limiting enzyme for cholesterol synthesis (Olender & Simon 1992). The location of DHCR24 on ER seems to be required for its function of catalyzing desmosterol to cholesterol, because the serial process of cholesterol synthesis occurs around the cytoplasmic sides of the ER membrane. Whether the location of DHCR24 on ER is also required for its ROS scavenging function has been poorly studied. What is the orientation of DHCR24's FAD-binding domain, which is important for its ROS scavenging function, on the ER membrane? Is the location of DHCR24 on the ER membrane necessary for its ROS scavenging function, especially for ER-generated ROS? To explore these issues, we studied the topological structure of DHCR24 on the ER membrane using the fluorescent protease protection (FPP) method in the present work.

The FPP assay is a recently developed method for investigating the topology of membrane proteins and has been successfully used in several studies (Lorenz *et al.* 2006, Cantero-Recasens *et al.* 2010, Hailey *et al.* 2010). This assay uses the restricted proteolytic digestibility of fluorescent protein (FP)-tagged TM proteins to indicate their intramembrane orientation. In this study, we generated three constructs

driving the expression of N-terminally DsRed-tagged or C-terminally EGFP-tagged full-length DHCR24 or N-terminally DsRed-tagged TM deleted DHCR24. We found that DHCR24 is located on the ER membrane with an N-terminal luminal/C-terminal cytoplasmic orientation.

Materials and methods

Plasmid construction

The plasmids for the FPP assay were constructed as follows. For the expression of DHCR24-EGFP fusion protein, the entire coding sequence of human *DHCR24* cDNA (GenBank accession no. NM_014762.3) was amplified by PCR using sense primer 5'-CTCGAGGC-CACCATGGAGCCCGCCGTGTGCTGGCC-3' and anti-sense primer 5'-GAATTCGGTGCCTGCCGGCC-TTGCAGATCTTGTC-3' (XhoI site, translation start site, and EcoRI site are underlined). A Kozak sequence was introduced in the sense primer. The stop codon was deleted in the anti-sense primer. The amplified cDNA was cloned into the XhoI-EcoRI site of plasmid pEGFP-N1 (Clontech-Takara Bio, Otsu, Japan). For the expression of DsRed-DHCR24 fusion protein, the entire *DHCR24* cDNA was amplified by PCR using sense primer 5'-CTCGAGCTGAG CCC GCC GTG TCG CTG GCC-3' and anti-sense primer 5'-GA A TTCTCAGTGCTGGCGGCCTTGCAGATCTTGTC-3' (XhoI site and EcoRI site are underlined). The start codon was deleted in the sense primer. The amplified cDNA was cloned into the XhoI-EcoRI site of plasmid pDsRed-monomer-C1 (Clontech-Takara Bio). For the expression of DsRed-DHCR24 without the predicted TM domain (DsRed-DHCR24 TM (-)), *DHCR24* cDNA with the 59–514 coding sequence deleted was amplified by PCR using sense primer 5'-CTCGAGCTAAGCTCAG-CAGCGCTCCGCGCCTG-3' and the same anti-sense primer as for *DsRed-DHCR24* cDNA. The amplified TM domain-deleted cDNA was then cloned into the XhoI-EcoRI site of plasmid pDsRed-monomer-C1. The adenovirus expressing DHCR24-myc was constructed as described previously (Lu *et al.* 2008). All of the primers were designed based on the GenBank *Homo DHCR24* mRNA sequence (GenBank accession no. NM_014762.3). Details of localization of the template sequences of each primer in the *DHCR24* mRNA are shown in Supplementary Figure 1, see section on supplementary data given at the end of this article.

Cell culture and transfection

N2A cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM/F12 (high glucose) supplemented with 10% fetal bovine

serum. We performed transient transfection using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. For imaging, we grew the cells on glass coverslips and analyzed them 48 h after transfection.

Western blotting analysis

For western blotting analysis, we grew equal amounts of N2A cells and transfected them in 6-well plates. Forty-eight hours after transfection, the cells were harvested and 30 µg/lane of each sample was then subjected to western blot analysis using the polyclonal anti-DHCR24 antibody (which recognizes the C-terminus), prepared as described previously (Lu *et al.* 2008), and mouse monoclonal anti-immunoglobulin heavy chain binding protein (Bip)/GRP78 antibody purchased from BD Biosciences (Bedford, MA, USA).

Florescence microscopy and imaging

We performed confocal laser scanning microscopy using Zeiss LSM 510 confocal systems. All images were taken with a 40/1.4 numerical aperture oil differential interference contrast Plan-Neofluar (Carl Zeiss, Jena, Germany) using a 488 nm Ar/Kr laser line or a 543-HeNe laser line. Scanning speed and laser intensity were adjusted to avoid photobleaching of the fluorophores and damage to the cells. The microscope was equipped with a microenvironmental chamber to maintain physiological conditions. For quantification of fluorescence intensities, nonsaturated images were taken with a fully open pinhole. For multichannel imaging, each fluorescent dye was imaged sequentially in the frame-interlace mode to eliminate cross talk between the channels. All image processing was performed using the Zeiss LSM 510 image examiner software.

Measurement of ROS production

Intracellular ROS levels were measured using a fluorescent dye technique (Lu *et al.* 2011). N2A cells were cultured on glass coverslips and were treated for 30 min with 20 µM 2',7'-dichlorofluorescein diacetate (H₂DCFDA; Molecular Probes, Eugene, OR, USA) in PBS. The coverslips were fixed and mounted. For the detection of H₂DCFDA fluorescence, the main beam splitter for excitation, the secondary beam splitter for emission, and barrier filter were 488, 570, and 505 nm long pass respectively. Several images were captured with the same set of optical parameters. The densitometric analysis was performed using Multi Gauge Software in LAS-1000 (Fuji Film, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed with ANOVA followed by Bonferroni's multiple *t*-test, and a *P* value < 0.05 was considered statistically significant.

Results

Prediction of membrane topological structure of DHCR24 and identification of fusion protein expression for the FPP assay

The *DHCR24* gene encodes a polypeptide of 516 amino acids with a potential N-terminal signal peptide, and at least one putative TM segment, predicted by the software. The amino acid sequence of human DHCR24 (seladin-1) was retrieved from GenBank/EBI Data Bank (entry name: DHCR24 HUMAN; accession number: Q15392). The primary transcription sequence (516 residues) was submitted to the Signal IP 3.0 Server to predict the cleavage site of the signal peptide. Predictions show the highest probability of cleavage between Gly22 and Leu23 leading to a putative functional protein of 494 residues. Given the known anchoring of DHCR24 to the membranes of the ER, the putative functional protein of 494 residues of DHCR24 was also submitted to TMPred (Hofmann & Stoffel 1993), which defines the length and position of the TM segments. The predicted result recognized the first 33 residues (Leu23–Trp55) as a plausible membrane segment (data not shown). This result is in accordance

Figure 1 Bioinformatical analysis of the membrane topology of DHCR24 and constructs for FPP. (A) Diagram of the probable topology of DHCR24 based on predictions obtained using TMPred. *In silico* predictions of potential transmembrane segments (black rectangles) of DHCR24 predict two possible models of the topological structure of DHCR24 on the ER membrane. The gray rectangles represent the predicted FAD-binding domain. (B) Schematic of fluorescent protein (FP)-tagged DHCR24 constructs. TM, transmembrane. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0132>.

with previous studies and is understandable given that the first segment represents a kind of peduncle, which protrudes from the whole enzymatic structure, seeming well-suited to anchor the protein to the membrane bilayer.

Based on the bioinformatics prediction, we propose two models of the topological structure of DHCR24 on the ER membrane. As shown in Fig. 1A, the potential structures of DHCR24 on the ER membrane seem to be either a C-terminal luminal/N-terminal cytoplasmic orientation or an N-terminal luminal/C-terminal cytoplasmic orientation. Note that there are also two possible locations of the FAD-binding domain: either in the cytoplasm or in the luminal space. In order to study the topological structure of DHCR24 using the FPP assay, we attached FPs to the C or N-terminus of DHCR24 (Fig. 1B). DHCR24–EGFP has EGFP fused to the C-terminus of DHCR24, while DsRed–DHCR24 has DsRed fused to the N-terminus. DsRed–DHCR24 TM (–) has DsRed fused to the N-terminus of the TM domain-deleted DHCR24 mutant. The plasmid expressing DsRed–DHCR24 TM (–) was constructed to confirm that DHCR24 is a single-spanning membrane protein as predicted.

The expression of these fusion proteins after transfection of plasmids into N2A cells was confirmed using confocal microscopy. As shown in Fig. 2A and B, both control FPs, EGFP and DsRed, are mainly located in the cytoplasm (Fig. 2A), whereas DHCR24–EGFP and DsRed–DHCR24 were located entirely around the nucleus (Fig. 2B left column). Immunocytochemistry using antibodies against the ER marker calreticulin revealed that the red fluorescent signal representing calreticulin was well merged with the green signal representing DHCR24–EGFP, suggesting an ER location of DHCR24–EGFP (Fig. 2B top row). A similar result was observed when the red signal of DsRed–DHCR24 and the green signal of calreticulin were merged (Fig. 2B middle row). However, DsRed–DHCR24 TM (–) clearly showed a cytoplasmic localization, as shown in Fig. 2B (lowest row). These results suggested that the predicted single-spanning membrane structure of DHCR24 is correct, which led us to use the FPP assay for the topological study. Taken together, these results demonstrate that the fusion proteins were expressed successfully in N2A cells.

This was further confirmed by immunocytochemistry using antibodies against DHCR24 (Fig. 2C). The endogenous expression of DHCR24 indicated by the white arrow was located around the nucleus, in accordance with our previous results revealing the ER location of DHCR24 (Greeve *et al.* 2000, Lu *et al.* 2008, Battista *et al.* 2009). The signals representing the overexpression of DHCR24 were also mainly around the nucleus and well merged with the signals

representing the FPs fused to DHCR24. Western blot analysis using anti-DHCR24 showed that the bands of both fusion proteins DHCR24–EGFP and DsRed–DHCR24 were about 90 kDa, similar to their expected size (Fig. 2D). All these data strongly demonstrate the successful expression of each fusion protein in N2A cells.

Figure 2 Identification of successful fusion protein expression in N2A cells. N2A cells were transfected with each of the plasmids for 48 h. (A) Images were directly obtained with a confocal laser microscope. (B and C) Immunocytochemical analysis was performed using the anti-calreticulin antibody or anti-DHCR24 (which recognizes the C-terminus) as the primary antibody and anti-rabbit IgG conjugated with Alexa Fluor 488 or 568 as the secondary antibody. Images were then obtained by confocal laser microscope and merged using Adobe Photoshop Software. Scale bar, 10 μ m. (D) The whole cell lysates either transfected with the plasmid or infected with adenovirus expressing myc-tagged DHCR24 were prepared and subjected to western blot using antibodies against DHCR24 (C-terminus) and Bip as the primary antibodies. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0132>.

Topological analysis of DHCR24 using the FPP assay demonstrated the location of DHCR24 on the ER membrane with an N-terminal luminal/C-terminal cytoplasmic orientation

Based on the principle of FPP, we studied the membrane topology of DHCR24 to identify the orientations of its N-terminus and C-terminus. The cells expressing FPs were briefly exposed to trypsin after plasma membrane permeabilization by digitonin. If the FP moiety on the expressed fusion protein faces toward the cytoplasm, its fluorescent signal should be lost. Conversely, if its FP moiety faces toward the luminal space of the ER, then its signal should persist. First, we established the conditions for the FPP assay in our experimental system. We found that digitonin treatment at a concentration of 40 μM for 5 min induced almost complete loss of the cytoplasm-localized control EGFP (Fig. 3A), suggesting that these conditions were suitable for the analysis.

First, we coexpressed the control DsRed and DHCR24–EGFP in the cells and performed the FPP assay (Fig. 3B). We observed the changes in red and green fluorescent signal intensities in the same cell. The red fluorescent signal representing DsRed quickly decreased after digitonin treatment, suggesting that the plasma membrane was successfully permeabilized. The green fluorescent signal from DHCR24–EGFP in the same cells was still observed after digitonin exposure, but quickly decreased after trypsin digestion, suggesting that the EGFP moiety on the DHCR24 fusion protein faces toward the cytoplasm. If so, we can speculate that the DsRed moiety on DsRed–DHCR24 should face toward the luminal space of the ER, and this was confirmed using an FPP assay with coexpression of EGFP and DsRed–DHCR24 (Fig. 3C). Fluorescence from EGFP quickly decreased after digitonin permeabilization, suggesting that the cell membrane was well permeabilized. The red fluorescent signal representing DsRed fused to DHCR24 did not change even after trypsin exposure, suggesting that the DsRed moiety faces toward the ER lumen where it is protected from trypsin digestion.

To further demonstrate the orientation of the N-terminus and C-terminus of DHCR24 in the ER lipid bilayer, we coexpressed DHCR24–EGFP and DsRed–DHCR24 in the cells and performed the FPP assay again. As shown in Fig. 4A, both green and red signals persisted after digitonin treatment under the same FPP assay conditions as in Fig. 3. Trypsin digestion induced the rapid decrease of green fluorescence from DHCR24–EGFP. By contrast, the red fluorescence from DsRed–DHCR24 did not show any substantial change during the treatment. This result was in accordance with that shown in Fig. 3. Based on these results, we can conclude that the membrane topology of DHCR24 on

ER is an N-terminal luminal/C-terminal cytoplasmic orientation (Fig. 4B). The FAD-binding domain should be in the C-terminal end of DHCR24, which faces toward the cytoplasm. This result is similar to the membrane topology of HMG CoA reductase, which is

Figure 3 Topological analysis of DHCR24 on the ER membrane using the FPP assay. (A) Digitonin treatment permeabilizes the plasma membrane and releases first cytosolic and later nucleoplasmic EGFP. N2A cells expressing EGFP were treated with 40 μM digitonin. Images were taken before and after digitonin application at the indicated time points. Scale bar, 10 μm . (B) FPP assay using DsRed- and DHCR24–EGFP-overexpressing cells. N2A cells expressing DsRed and DHCR24–EGFP were subjected to the FPP assay. Images were taken before and after 5 min treatment with 40 μM digitonin and 4 mM trypsin as indicated. Scale bar, 10 μm . (C) FPP assay using EGFP- and DsRed–DHCR24-overexpressing cells. Scale bar, 10 μm . Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0132>.

Figure 4 The FPP assay reveals the topology of the ER membrane protein DHCR24. (A) N2A cells coexpressing DHCR24-EGFP and DsRed-DHCR24 were treated with 40 μ M digitonin for 5 min. Images were taken before and after digitonin application. Scale bar, 10 μ m. (B) Diagram of the topology of DHCR24 on the ER membrane. (C) Diagram showing localization of TM domain-deleted DHCR24 in the cytoplasm. The predicted caspase cleavage sites are indicated by the arrows. The FAD-binding domains are represented by the shaded boxes. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0132>.

the rate-limiting enzyme during *de novo* cholesterol synthesis with its catalytic domain on its C-terminus facing the cytoplasm (Olender & Simon 1992). Note that the predicted caspase cleavage sites are expected to be located on the side facing the cytoplasm in this model. However, TM domain-deleted DHCR24 localized to the cytoplasm rather than the ER membrane (Fig. 2B, lowest row), which is represented by the diagram in Fig. 4C. In this case, the FAD-binding domain and the caspase cleavage sites are still present in TM domain-deleted DHCR24, suggesting that TM domain-deleted DHCR24 could still exert its anti-apoptotic function despite its cytoplasmic localization. This possibility is proved by the next experiments.

Localization of DHCR24 to the ER membrane is not necessary for its ROS scavenging function at the cellular level

De novo cholesterol biosynthesis occurs in cytoplasm and ER and most enzymes involved in cholesterol biosynthesis are present in both cytoplasm and ER. It is evident that the topology of DHCR24 on the ER membrane with its FAD-binding domain facing the cytoplasm is essential for its enzymatic function for cholesterol biosynthesis because its substrates, desmosterol and others, are produced around the ER membrane facing the cytoplasm. We previously demonstrated that the FAD-binding domain is essential for the ROS scavenging function of DHCR24. Is the location of DHCR24 on the ER membrane also required for ROS scavenging? We next measured intracellular ROS using the fluorescent probe H₂DCFDA. As shown in Fig. 5A, the green fluorescent signal representing ROS levels was strongly decreased in both DsRed-DHCR24- and DsRed-DHCR24 TM (-)-overexpressing cells after the H₂O₂ exposure for 60 min, compared with that in the DsRed-overexpressing cells. These decreases were significant (Fig. 5B). We could also identify the different location of full-length DHCR24 (in the ER) compared with the TM domain-deleted DHCR24 (in the cytoplasm), which is consistent with the data shown in Fig. 2C. Taken together, these results demonstrate that TM domain-deleted DHCR24 exerts its ROS scavenging function at the cellular level despite not being localized to the ER membrane.

The DHCR24 content on the ER is decreased upon exposure to oxidative stress accompanied by activation of caspase

A previous study has demonstrated that DHCR24 is a substrate for caspases (Greeve *et al.* 2000). Our data suggested that its predicted caspase cleavage sites should be in its C-terminus, away from its TM domain and facing toward the cytoplasm. To further test this possibility, we coexpressed the DHCR24-EGFP and DsRed-DHCR24 fusion proteins in N2A cells and applied an oxidative stress by H₂O₂ exposure. First, we tested the activation of caspase upon H₂O₂ exposure. As shown in Fig. 6A and B, fluorescence microscopy revealed that caspase-3 was activated after H₂O₂ exposure for 1 and 3 h, suggesting that H₂O₂ treatment activated the apoptotic molecular pathway. Under these conditions, we observed the changes in fluorescent signal intensity (Fig. 6C and D). The green signal representing DHCR24-EGFP was strong and concentrated around the nuclei in the control cells (0 h). However, the signal became very weak with reduced presence around the nuclei after exposure to

Figure 5 The TM domain-deleted DHCR24 mutant can also scavenge intracellular ROS. N2A cells expressing DsRed, DsRed-DHCR24, or DsRed-DHCR24 TM (-) were exposed to 1 mM H₂O₂ for the indicated times and the cells were incubated for 10 min with H₂DCFDA. Cell images were obtained with a confocal laser microscope. Scale bar, 100 μ m. (B) Fluorescent intensities of H₂DCFDA are expressed as fold increase in the control level. Mean $\pm$ s.d. (n=20). **P* < 0.05 vs the control (0 h) level. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0132>.

H₂O₂ for 3 h. The reduction in fluorescence-integrated density of the green signal was significant (Fig. 6D). In contrast, the red signal representing DsRed-DHCR24 in the same cells was not significantly changed. This result suggested that DHCR24-EGFP was cleaved by activated caspase-3 and the EGFP fused to the C-terminus diffused throughout the cytoplasm under H₂O₂ stimulation. Of course, DsRed-DHCR24 was also cleaved at its cleavage sites facing the cytoplasm, and the remaining part of DsRed-DHCR24 with DsRed fused to the N-terminus persisted on the ER membrane with the TM domain; thus, we did not observe a change in red signal intensity.

Please note that the apoptotic cells indicated by white arrows revealed concentrated nuclei, which were identified by Hoechst 33258 staining. Most of the apoptotic cells expressed lower levels of the DHCR24 fusion proteins. In contrast, the cells overexpressing the DHCR24 fusion proteins are protected from apoptosis. Taken together, these results again demonstrated that DHCR24 can protect cells from oxidative stress-induced apoptosis, which involves the activation of caspase, which cleaves DHCR24.

Discussion

Correct localization and topology are crucial for understanding the cellular function of a membrane protein. This study demonstrates for the first time that DHCR24 is localized to the ER membrane with an N-terminal luminal/C-terminal cytoplasmic orientation. First, we demonstrated that DHCR24 is a protein with a single membrane-spanning topology and that its N-terminal TM domain is essential for its localization to the ER membrane (Figs 1 and 2B). This observation led us to use the FPP assay to explore the topology of DHCR24 on the ER membrane. A series of FPP analyses were performed and all the results strongly demonstrated the presence of DHCR24 on the ER membrane with an N-terminal luminal/C-terminal cytoplasmic orientation (Figs 3 and 4).

Based on this topological analysis of DHCR24, we can draw the conclusion that the FAD-binding domain of DHCR24 is located in its cytoplasmic side. Thus, DHCR24 should also scavenge H₂O₂ even when it is present in the cytoplasm, because H₂O₂ can pass through the plasma membrane and diffuse throughout

Figure 6 DHCR24-EGFP fluorescence is decreased in response to oxidative stress, accompanied by the activation of caspase-3. (A) At the indicated time after exposure to 1 mM H₂O₂, cells cultured on the coverslips were fixed and subjected to immunocytochemistry with a primary antibody against rabbit active caspase-3 and a secondary antibody against rabbit IgG conjugated with Alexa Fluor 488. Representative images are shown. Bar, 10 μ m. (B) Fluorescent intensities are expressed as fold increase in the control level. Mean $\pm$ s.d. (n=20). * P <0.05 vs the control (0 h) level. (C) N2A cells coexpressing DHCR24-EGFP and DsRed-DHCR24 were treated with 1 mM H₂O₂ and subjected to Hoechst staining. Images were obtained with a confocal microscope and then merged using Adobe Photoshop. Scale bar, 10 μ m. (D) Fluorescent intensities are expressed as fold increases in the control level. Mean $\pm$ s.d. (n=20). * P <0.05 vs the control (0 h) level. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0132>.

the cytoplasm freely. Our data confirmed this possibility (Fig. 5). Deletion of the TM domain resulted in a change in DHCR24 localization from ER to the cytoplasm. Under these conditions, the DHCR24 mutant still demonstrated scavenging activity of intracellular ROS generated from H₂O₂, suggesting that the localization of DHCR24 to the ER membrane is not essential for its ROS scavenging function. Our data showed that fluorescence signal intensity of DHCR24-EGFP was decreased in response to H₂O₂, accompanied by activation of caspase-3 (Fig. 6). This result suggests that DHCR24-EGFP was cleaved by caspase-3, possibly at the predicted caspase cleavage sites located in the cytoplasmic side of DHCR24, where caspase-3 is activated during the early stages of apoptosis. The green fluorescent signal representing the EGFP moiety was lost, which might be explained by the cleaved part of DHCR24-EGFP diffusing through the cytoplasm so that its density became so low as to be undetectable. This is supported by immunocytochemical analysis using an antibody against the C-terminus of DHCR24 to identify endogenous DHCR24 levels (Supplementary Figure 2, see section on supplementary data given at the end of this article). The results revealed that the endogenous DHCR24 was initially diffused in the cytoplasm during the early stages after H₂O₂ exposure, and its expression then increased after H₂O₂ stimulation for 3 h. The upregulation of DHCR24 in response to H₂O₂ has also been seen in several previous studies (Wu *et al.* 2004, Kuehnle *et al.* 2008, Battista *et al.* 2009). In this study, the expression of DHCR24-EGFP fusion protein is driven by the CMV promoter in the plasmid pEGFPN1, which is not regulated by the same mechanisms as endogenous DHCR24, so we failed to

observe the upregulation of DHCR24-EGFP, rather than its dispersal from the ER membrane (Fig. 6). DsRed-DHCR24 might also have been decreased because it also has caspase-cleaving sites. However, the red fluorescent signal representing DsRed-DHCR24 was not changed, which could be explained by the presence of the remaining part of DsRed-DHCR24 with its N-terminal red fluorescent DsRed moiety on the luminal side of the ER membrane.

Based on our topological analysis of DHCR24, the characterization of a model of the DHCR24 enzyme is of relevance in the light of recent experimental data that suggest a dual role of DHCR24/seladin-1 in the control of cell survival, exerting an acute response linked to cholesterol biosynthesis and a late response with a cholesterol-independent anti-apoptotic activity, possibly by interaction with the apoptotic protein p53 (Kuehnle *et al.* 2008). We observed the cholesterol-independent anti-apoptotic activity of DHCR24, demonstrated by the finding that DHCR24 still exerts its ROS scavenging function even when it is located in the cytoplasm (Fig. 5), where it should not catalyze cholesterol biosynthesis because of the limited source of substrate.

DHCR24 has been found to be able to translocate to the nucleus in response to stress stimuli in fibroblasts, suggesting that a soluble isoform of the protein could be generated under appropriate conditions (Battista *et al.* 2009). We could not observe the translocation of DHCR24 into the nucleus, which might be because of limitations of the methods employed. In our case, the remaining middle part without EGFP after cleavage by caspase-3 might translocate into the nucleus and interact with p53, by which DHCR24 exerts anti-apoptotic function, as has

been previously reported (Wu *et al.* 2004, Battista *et al.* 2009). This is supported by evidence that there is amino acid homology between two possible caspase cleavage sites and the MDM2 binding site to p53, but further evidence is needed.

Taken together, these results suggest that DHCR24 functions as a ROS scavenger even after being cleaved by activated caspase-3, which might provide a new insight for understanding the mechanisms of the cell-protective function of DHCR24.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.101530/JME-11-0132>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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