〈Regular Article〉

The efficient detection of membrane protein with immunoblotting: lessons from cold-temperature denaturation

Kei MIYANO[1),*], Shuichiro OKAMOTO[1),*], Mizuho KAJIKAWA[2)]
Chikage KAWAI[1)], Tomoka KANAGAWA[3)], Sayuri TOMINAGA[3)]
Akira YAMAUCHI[1)], Futoshi KURIBAYASHI[1)]

1) Department of Biochemistry, Kawasaki Medical School
2) Laboratory of Microbiology, Showa Pharmaceutical University
3) Second Year Medical Student in fiscal year of 2020, Kawasaki Medical School
** These authors contributed equally to this work.*

ABSTRACT Transmembrane proteins play essential roles in cell signaling, transport of membrane-impermeable molecules, cell-cell communication, and cell adhesion. Our recent work demonstrated that reactive oxygen species-generating NADPH oxidase 4 (Nox4), a protein with multiple transmembrane domains, is involved in cell migration by stabilizing vascular endothelial growth factor receptor 2 (VEGFR-2), a single-span transmembrane protein. During this study and with further verification, we developed a simple method to prepare protein samples without aggregating these membrane proteins for SDS-PAGE, immunoblotting, and deglycosylation assay. We found that heating was unnecessary for protein denaturation for SDS-PAGE and deglycosylation assay. Also, the detectable amounts of VEGFR-2 and Nox4 were increased in the sample treated at 4℃ compared with the sample treated at 98℃ . Moreover, the *N*-glycan of VEGFR-2 was digested by glycosidase at reaction temperature 4℃ .

doi：10.11482/KMJ-E202147013 *(Accepted on January 18, 2021)*

Key words：*Membrane protein, Endoglycosidase, Immunoblotting, SDS-PAGE, VEGFR-2, NADPH oxidase 4*

INTRODUCTION

Membrane proteins are generally classified and named into the following three types: "Type I" membrane protein, the N-terminus is located outside the cell; "Type II" membrane protein, the N-terminus is located inside the cell; and "multiple" transmembrane protein. All three types have at least one transmembrane hydrophobic helix. The helix

Corresponding author
Kei Miyano
Department of Biochemistry, Kawasaki Medical School, 577 Matsushima, Kurashiki, 701-0192, Japan
Phone : 81 86 462 1111
Fax : 81 86 464 1199
E-mail: kei-miyano@med.kawasaki-m.ac.jp

Shuichiro Okamoto
Department of Biochemistry, Kawasaki Medical School, 577 Matsushima, Kurashiki, 701-0192, Japan
Phone : 81 86 462 1111
Fax : 81 86 464 1199
E-mail: shuokamoto@med.kawasaki-m.ac.jp

often makes protein detection by immunoblotting challenging because its hydrophobicity causes protein aggregation.

Recently, we found that reactive oxygen species-generating Nox4 enhanced the directed migration of endothelial cells by maintaining surface VEGFR-2 levels in the endoplasmic reticulum[1]. Nox4 is a transmembrane protein predicted to have 6 transmembrane regions[2]. VEGFR-2 is a type I membrane protein[3]. During our research, it was necessary to prevent protein aggregation to quantify the amount of these membrane proteins by immunoblotting accurately. This paper aims to provide methodologic details and further verification regarding the denaturation of transmembrane protein for SDS-PAGE, immunoblotting, and deglycosylation assay.

MATERIALS AND METHODS

Reagents and materials

All general ultrapure-grade reagents were purchased from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemicals Industries (Tokyo, Japan), or Sigma-Aldrich Japan (Tokyo, Japan) unless otherwise stated. 2 × Laemmli sample buffer (catalog #1610737) was purchased from Bio-Rad (Tokyo, Japan).

Cells and cell culture

EA.hy926 cells were purchased from ATCC, and CHO-K1 cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). The EA.hy926 cells were cultured as previously described[1]. The CHO-K1 cells were maintained in Nutrient Mixture F-12 [HAM] medium (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Sigma-Aldrich) in a humidified incubator at 37℃ with 5% (v/v) CO_2.

Plasmids preparation and Plasmid transfection

pcDNA3.1-human Nox4 was a gift from Karl-Heinz Krause (Addgene plasmid #69352; http://n2t.net/addgene:69352; RRID: Addgene_69352). The cDNA for the human $p22^{phox}$ was prepared by reverse transcribing the mRNA from EA.hy926 cells into cDNA and then cloning the cDNA into pcDNA3.1.

The plasmids were transfected into CHO-K1 cells using the Viofectin™ Transfection Reagent (Viogene). The cells were seeded in 6-well plates at 3.5×10^5 cells per well 24 h before transfection. Before transfection, the plates were washed twice with PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.4). Then, 1 ml of serum-free F-12 medium was added. A volume of 7.5 μl of Viofectin was diluted with 25 μl of serum-free F-12 medium. An amount of 1 μg of pcDNA3.1-Nox4 and 0.1 μg of pcDNA3.1-$p22^{phox}$ were diluted with 25 μl of serum-free F-12 medium. The diluted Viofectin and DNA were mixed and incubated at room temperature for 15 min. The mixtures were added to the plates. The plates were incubated for 2 h at 37℃ in a humidified 5% (v/v) CO_2 atmosphere. In order to remove the remaining mixtures, the culture medium was replaced with fresh F-12 medium supplemented with 10% (v/v) FBS. Then, the transfected cells were cultured for 48 h.

Sample preparation for immunoblotting and glycosidase treatment

The EA.hy926 cells were cultured in 6-well plates in a confluent state for 48 h. The plates of EA.hy926 cells and transfected CHO-K1 cells were washed twice with ice-cold PBS. Next, 50 μL of lysis buffer (20 mM Tris-Cl (pH 7.4 at 4℃), 150 mM NaCl, 1% (w/v) Triton X-100, 1% (v/v) protease inhibitor cocktail) was added to each well. The cells were scraped with a scraper and were pipetted into a microcentrifuge tube. The cell lysates were mixed by pipetting ten times and vortexing, and then incubated for 1 h at 4℃. The lysate was centrifuged at 10,000 × g for 20 min at 4℃. The supernatant of

each microcentrifuge tube was collected, frozen in small aliquots in liquid nitrogen, and store at -80℃ until the immunoblotting experiments.

For TCA (trichloroacetic acid) treatment, after trypsin harvest of CHO-K1 cells or EA.hy926 cells from 6-well plates, the cells were washed three times with ice-cold PBS and resuspended in 50 μL of PBS. 5 μL of 100% (w/v) TCA was added to the suspension. The mixtures were mixed by pipetting ten times and vortexing, and then incubated for 1 h at 4℃. The mixtures were centrifuged at 10,000 × g for 15 min at 4℃. The precipitates were resuspended to 50 μL of UTM:sample buffer (10 M Urea, 2% (w/v) Triton X-100, 20% (v/v) 2-mercaptoethanol in 1 × Laemmli sample buffer) and then lysed by sonication. Lysates were titrated with 2 M Tris until the color of bromophenol blue changed from yellow to blue.

Immunoblotting procedure

The schematic illustration for sample preparation was included (Fig. 1A). The 2 × Laemmli sample

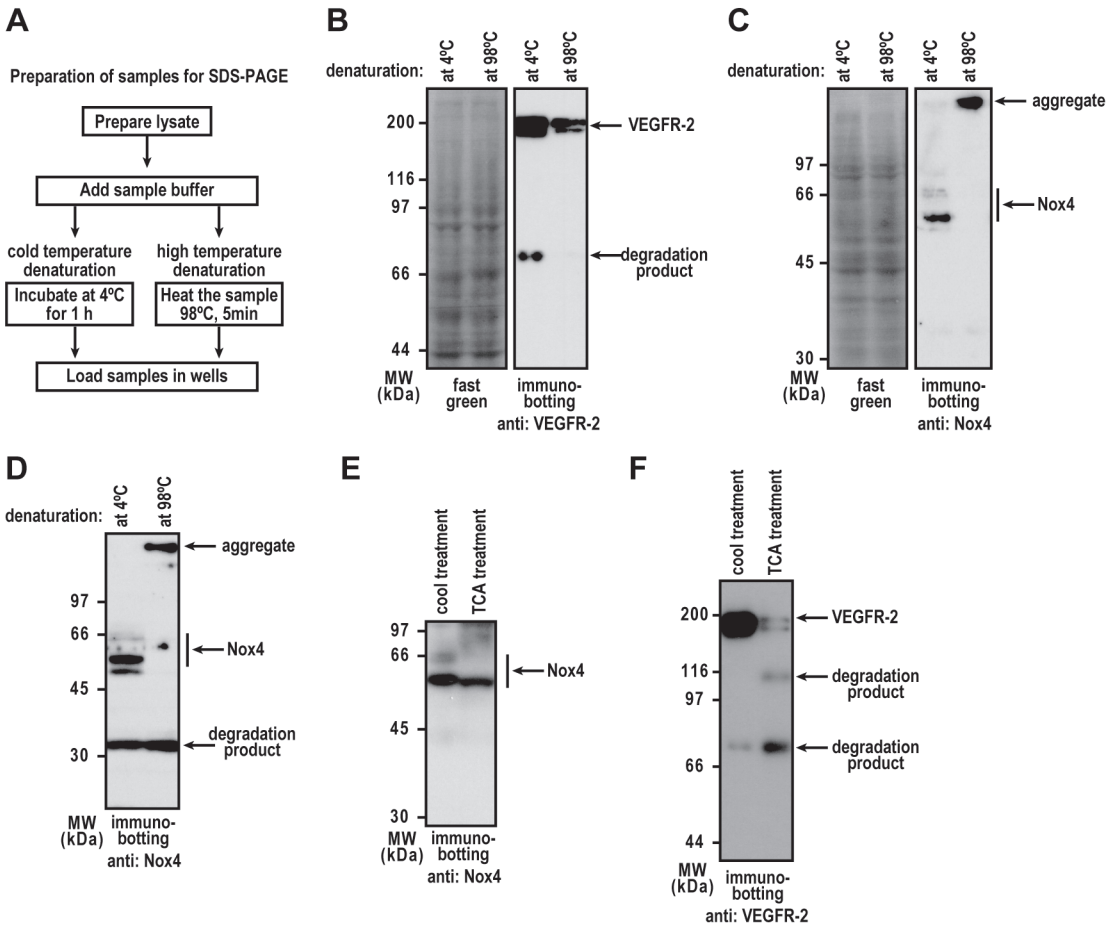

Fig. 1. The cold denaturation of transmembrane proteins.
A, The schematic illustration of the experiment.
B and F, The level of VEGFR-2 protein visualized by immunoblotting. After the indicated treatment of cell lysate (EA.hy926 cells), protein levels of VEGFR-2 were estimated by immunoblot analysis with the anti-Nox4 polyclonal antibody.
C–E, The level of Nox4 protein visualized by immunoblotting. CHO cells (C and E) were cotransfected with the indicated plasmids: pcDNA3.1-human Nox4 and pcDNA3.1-human $p22^{phox}$. After the indicated treatment of cell lysate (C and E, CHO cells; D, EA.hy926 cells), protein levels of Nox4 were estimated by immunoblot analysis with the anti-Nox4 polyclonal antibody.

buffer (Bio-Rad catalog #1610737), including 2% (v/v) 2-mercaptoethanol, was added to cell lysates or the glycosidase-treated sample (see *Glycosidase treatment* for sample preparation). The mixtures were incubated at 4℃ for 1 h (cold temperature denaturation). The denatured samples were loaded into a polyacrylamide gel; an ice-cold running buffer was used during the electrophoresis.

PVDF membrane was soaked in methanol for 1 min and equilibrated in the transfer buffer containing 6.06 g of Tris, 28.8 g of glycine, and 200 ml of methanol in 2L of distilled H_2O. The polyacrylamide gel was also equilibrated in the transfer buffer and transferred to the PVDF membrane at 50 V for 2 h using Wet type blotting device (Criterion™ blotter, BioRad). During the transfer, the transfer buffer was maintained at 4℃. After the transfer, the PVDF membrane was temporarily stained with the FAST green, which consisted of 0.1% (w/v) FAST green, 30% (v/v) ethanol, and 10% (v/v) acetic acid, and then rinsed with distilled water to remove the background staining. The membrane was incubated in a blocking buffer of 5% (w/v) skim milk and 0.05% (v/v) Tween in TBS (20 mM Tris-Cl (pH 7.4), 150 mM NaCl) at room temperature for 30 min with shaking. Then, the membrane was incubated with a 1:1,000 diluted anti-Nox4 antibody (Gene Tex, NOX4 antibody [N3C3]) or anti-VEGFR-2 (Cell signaling Technology, 55B11 mAb #2479) in antibody dilution solution (Dream Realization & Communication, DRC-W00H250) at 4℃ overnight. Afterward, the membrane was incubated a 1:5,000 diluted secondary antibody conjugated with horseradish peroxidase in 5% (w/v) skim milk in TBS at room temperature for 30 min. The protein bands on the blots were detected using ImmunoStar® Zeta or LD (FUJIFILM Wako pure chemical corporation).

Glycosidase treatment

A volume of 20 μl of the lysate from EA.hy926 cells was incubated at 25℃ or 4℃ for 1 h with or without 1 μl of peptide N-Glycosidase F (PNGase F) (New England BioLabs, P0704S) or 1 μl of Endoglycosidase H (Endo H) ((New England BioLabs, P0702S).

RESULTS

Cold-temperature denaturation for immunoblotting

We investigated whether heat denaturation was required to detect VEGFR-2, a type I membrane protein, by immunoblotting (Fig. 1A). VEGFR-2 protein was observed at its expected position of 200 kDa with cold temperature denaturation (4℃ lane), i.e., without heat denaturation at 98℃. Compared to the sample incubated at 4℃ for 1 h, the detectable amount of VEGFR-2 was reduced in the sample treated at 98℃ (Fig. 1B); the amount of protein in the two samples that had been transferred to the PVDF membrane was comparable. A second band of significant intensity had an apparent molecular weight of 75 kDa; it was derived from the degradation of VEGFR-2. This second band also decreased due to heat treatment (Fig. 1B). This 75-kDa degradation product likely consists of a transmembrane domain and cytosolic domain because it is recognized by the antibody against the cytosolic domain of VEGFR-2, and the molecular weight indicates that the product contains transmembrane and cytosolic domains.

For Nox4, which contains multiple transmembrane domains, the heat treatment might have a more adverse effect on protein detection. Because Nox4 forms a complex with $p22^{phox}$ to function as a ROS-producing oxidase[4], we co-expressed Nox4 with $p22^{phox}$ in CHO-K1 cells, which do not express endogenous Nox4 and $p22^{phox}$. Indeed, the heat-treated Nox4 was not detected at the expected position according to its molecular weight on the gel (Fig. 1C). Instead, the Nox4 antibody recognized the protein at the top of the running gel, suggesting the aggregation of the Nox4 protein. The detectable

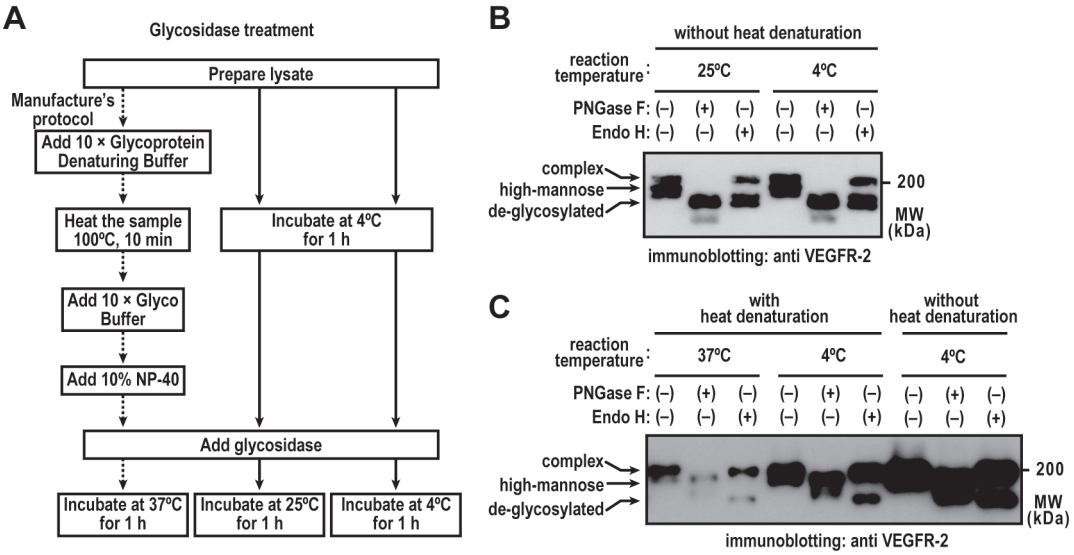

Fig. 2. The denaturation of integral membrane glycoprotein at the cold temperature.
A, The schematic illustration of the experiment. The lysate was denatured at 4℃ for 1 h; the manufacturer's protein denaturation protocol (indicated by the dotted arrow) was not used.
B and C, Examination of reaction temperature for *N*-glycan cleavage. VEGFR-2 in the cell lysate (from EA.hy926 cells) was digested with PNGase F or EndoH and subjected to SDS-PAGE, followed by immunoblot analysis with the anti-VEGFR-2 polyclonal antibody.

amount of Nox4 endogenously expressed in EA.hy926 cells was increased in the sample treated at 4℃ compared with the sample treated at 98℃ (Fig. 1D). Thus, it was found that cold denaturation is suitable for both endogenously expressed Nox4 and overexpressed Nox4.

In previous studies, it was shown that multiple transmembrane proteins were readily detected by TCA treatment of cells without heat denaturation[5]. Nox4 in the TCA-treated lysate was detected to the same extent as the cold denatured Nox4 (Fig. 1E). For VEGFR-2, the TCA treatment reduced the amount of full-length VEGFR-2 with an increase of the degradation products (Fig. 1F). Thus, given that the TCA treatment procedure is complicated and some proteins are degraded (Fig. 1F), the cold denatured method is simpler and easier to use.

Glycosidase reaction at cold temperature

According to the manufacturer's protocol, the sample must be treated with heat denaturation (Fig. 2A) and be deglycosylated by glycosidase at the reaction temperature of 37℃. We investigated whether the sugar chains of proteins in Triton X-100 extract (cell lysate) could be cleaved by glycosidase without heat denaturation to avoid sample aggregation. In addition, the reaction temperature of 25℃ or 4℃ was also examined. We previously found that VEGFR-2 existed as doublets in EA.hy926 cells[1]. The higher-molecular-weight band was Endo H-resistant and PNGase F-sensitive. In contrast, the lower-molecular-weight band was sensitive to Endo H and PNGase F. In other words, VEGFR-2 was expressed as a protein with high-mannose, localized in the endoplasmic reticulum, and a protein with complex *N*-glycan, localized in the plasma membrane, in EA.hy926 cells[1]. The *N*-glycan of VEGFR-2 could be digested with PNGase F and Endo H at 4℃ without heat denaturation (Fig. 2B). In the heat denatured sample, although VEGFR-2 was deglycosylated at the reaction temperature of 4℃, the detectable

amount of VEGFR-2 was reduced (Fig. 2C). This may be due to the aggregation of VEGFR-2 by heat denaturation. Furthermore, the reaction temperature of 37℃ made it more difficult to detect VEGFR-2 bands, suggesting VEGFR-2 protein aggregation progressed further during reaction with glycosidase.

DISCUSSION

Protein denaturation by heat treatment is required for immunoblotting of proteins and deglycosylation of glycoprotein. It is challenging to detect proteins with multiple transmembrane domains by immunoblotting because the heat treatment induces protein aggregation (Fig. 1). On the other hand, protein denaturation is caused by cooling the protein from room temperature to lower values[6, 7]. In the present study, we demonstrated that the cold treatment is more efficient than heat treatment or TCA treatment for denaturation of membrane proteins required for immunoblotting and deglycosylation.

We found that the heat treatment of proteins with the multiple transmembrane domains, such as Nox4, caused a more severe problem than type I membrane proteins, such as VEGFR-2. The difference between the two proteins is the size of their respective transmembrane regions. A standard transmembrane helix is composed of about 20 amino acid residues. VEGFR-2 is assumed to have 21 residues for its transmembrane helix[3], whereas Nox4 is assumed to have about 120 residues (because Nox4 is predicted to have 6 transmembrane regions)[2]. The hydrophobic helices are completely exposed by heat denaturation, likely causing protein aggregation. Thus, Nox4 might be more highly aggregated because of the length of its hydrophobic region. Consistent with this hypothesis, Nox4-derived aggregates were observed by immunoblotting (Fig. 1C). On the other hand, VEGFR-2 aggregates were not detected by the anti-VEGFR-2 antibody (Fig. 1B); the VEGFR-2 aggregate might not have migrated through the stacking gel.

Previously, it was shown that the TCA (trichloroacetic acid) treatment of cells was an effective sample preparation method for detecting phagocyte Nox2, which belonged to the Nox family, in the phagocyte by immunoblotting[5]. Neutrophils, rich in protease, often cause undesired proteolysis during sample preparation. Therefore, the TCA treatment is used to prevent proteolysis. This procedure not only avoids proteolysis but also prevents Nox2 aggregation and enables detection by immunoblotting. However, this procedure is a little complicated. In addition, the TCA treatment causes degradation of some membrane proteins (Fig. 1F). The method in our study is more straightforward because we only incubate the cell lysate with the Laemmli sample buffer at 4℃.

The intracellular localization of a membrane protein can be examined by the digestion of *N*-glycan with glycosidase. However, according to the manufacture's protocol, membrane proteins need to be denatured by heat. This study showed that heat denaturation was dispensable and the optimal reaction temperature was 4℃ because the reaction temperature of 37℃ made it more difficult to detect VEGFR-2 bands (Fig. 2C). Thus, we believe our method is useful in analyzing the localization of membrane proteins.

In summary, we show that protein denaturation by heat treatment is dispensable for immunoblotting of proteins and deglycosylation of glycoprotein. The cold treatment should be first selected as a denaturing condition for membrane proteins because the cold treated sample can be heat treated later.

ACKNOWLEDGMENTS

We are grateful to Aya Morihara (Kawasaki Medical School, Japan) for technical assistance and to the Central Research Institute of Kawasaki Medical School for technical support. This study was supported in part by JSPS KAKENHI grant

numbers JP17K08637 (KM), in part by the Wesco Scientific Promotion Foundation (KM), in part by the Ryobi Teien Memory Foundation (KM), and in part by Research Project Grants [nos. R01S-003 (KM)] from Kawasaki Medical School.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1）Miyano K, Okamoto S, Yamauchi A, Kawai C, Kajikawa M, Kiyohara T, Tamura M, Taura M, Kuribayashi F: The NADPH oxidase NOX4 promotes the directed migration of endothelial cells by stabilizing vascular endothelial growth factor receptor 2 protein. J Biol Chem. 2020; 295: 11877-11890. doi: 10.1074/jbc.RA120.014723.

2）Lambeth JD, Neish AS: Nox enzymes and new thinking on reactive oxygen: a double-edged sword revisited. Annu Rev Pathol. 2014; 9: 119-145. doi: 10.1146/annurev-pathol-012513-104651.

3）Holmes K, Roberts OL, Thomas AM, Cross MJ: Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition. Cell Signal. 2007; 19: 2003-2012. doi: 10.1016/j.cellsig.2007.05.013.

4）von Löhneysen K, Noack D, Jesaitis AJ, Dinauer MC, Knaus UG; Mutational analysis reveals distinct features of the Nox4-p22*phox* complex. J Biol Chem. 2008; 283: 35273-35282. doi: 10.1074/jbc.M804200200.

5）Yu D, Imajoh-Ohmi S, Akagawa K, Kanegasaki S: Suppression of superoxide-generating ability during differentiation of monocytes to dendritic cells. J Biochem. 1996; 119: 23-28. doi: 10.1093/oxfordjournals.jbchem.a021211.

6）Sanfelice D, Temussi PA: Cold denaturation as a tool to measure protein stability. Biophys Chem. 2016; 208: 4-8. doi: 10.1016/j.bpc.2015.05.007.

7）Privalov PL: Cold denaturation of proteins. Crit Rev Biochem Mol Biol. 1990; 25: 281-305. doi: 10.3109/10409239009090612.