Importance of conserved amino acids in transmembrane domains

of rhodopsin type GPCRs in their quality control and function

ロドプシン型GPCRの膜貫通領域に保存されたアミノ酸残基

の品質管理および機能における重要性に関する研究

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Abbreviations

BLT2, leukotriene B_4 type-2 receptor;

BSA, bovine serum albumin;

Doa10, degradation in the endoplasmic reticulum protein 10;

Dox, doxycyclin;

Endo-H, Endoglycosidase H;

ER, endoplasmic reticulum;

ERAD, endoplasmic reticulum-associated degradation;

GnRHR, gonadotropin-releasing hormone;

GPCR, G-protein coupled receptor;

HA, haemagglutinin;

HRD1, HMG-CoA reductase degradation protein 1

HRP, horseradish peroxidase;

mc-PAF, methylcarbamyl PAF;

MFI, mean fluorescence intensity;

NP-40, Nonidet P-40;

PAF, platelet-activating factor;

PAFR, platelet-activating factor receptor;

PE, phycoerythrin;

PNGase-F, *N*-Glycosidase F;

PBS, phospate-buffered saline;

RFU, relative fluorescence unit;

SA-HRP, streptavidin horseradish peroxidase;

S.D., standard deviations;

SDS, sodium dodecyl sulfate;

SNP, single nucleotide polymorphism;
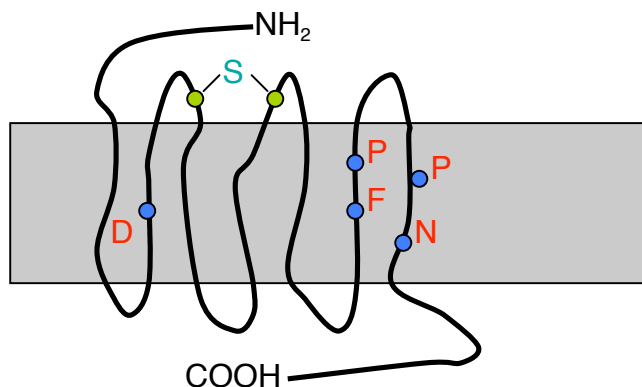
TM, transmembrane;

V2R, vasopressin V2 receptor;

WT, wild-type

Abstract

Several amino acid residues in the transmembrane (TM) domains of rhodopsin-type G-protein coupled receptors (GPCRs) are conserved. Here, I show that mutations in several of these residues interfere the export of GPCRs by the endoplasmic reticulum (ER), and ligands specific for these receptors have the potential to rescue the ER retention. In HeLa cells, replacement of several conserved residues in TM2, TM6, and TM7 by alanine resulted in a significant reduction of cell surface expression of the platelet-activating factor receptor (PAFR). In particular, the importance of the aspartic acid in TM2 and the proline in TM6 were confirmed in two other GPCRs, leukotriene B_4 type-2 receptor and GPR43. Although PAFRs containing these mutated residues, including D63A and P247A, accumulated in the ER, the cell surface expression of these receptors was facilitated by the addition of the PAFR ligands methylcarbamyl PAF or Y-24180. Because the Y-24180 treatment reduced the ubiquitination of the mutant PAFRs and increased the proportion of fully glycosylated forms, the augmentation of the cell surface receptor is due to the facilitation of ER export. While the surface-trafficked P247A mutant showed the potential to elicit an increase of intracellular Ca^{2+} by PAF, D63A mutants lacked this signaling ability. Taken together, my data indicate that deficiency of conserved amino acid residues in GPCRs inhibits their export from the ER, and that treatment

with pharmacological chaperones allows them to pass the quality control system in the

ER, even though some of them are dysfunctional receptors.

Introduction

Family 1 GPCR(1)   (Rhodopsin-type GPCR)
Retinal, odorants, catecholamines, adenosine, ATP, opiates, enkephalins, anandamide, *etc*
Blue circles indicate conserved amino acid residues in transmembrane domains.
Green circles indicate cystein residues.

Many G-protein coupled receptors (GPCRs) classified in the rhodopsin-type family

have several common residues and several structural motifs are reported(1, 2).

Numerous research groups have predicted that these residues are crucial for the

function of GPCRs, and several reports have demonstrated the significance of these

conserved residues for ligand recognition and/or GTPγS binding using membrane

preparations from cells expressing various mutant GPCRs (3-7), including

platelet-activating   factor   (1-*O-alkyl*-2-*acetyl-sn-glycero*-3-phosphocholine)   (PAF)

receptor (PAFR) (8-11).   They reported the importance of conserved residues, but in

many cases lack the validation of receptor expression on plasma membrane.   It is

possible that mutant GPCRs used in these reports are degraded in the endoplasmic

reticulum (ER) because of their aberrant structure.   In this paper I tried to focus on the

cell suraface expression of GPCRs mutated at several conserved residues, and studied

the effects of pharmacological chaperones.

I took particular note of common residues located in their seven transmembrane

helices.   Transmembrane (TM) domains of GPCRs exist on the cell surface and

GPCRs need to keep them in stable formation to couple with G-proteins and make

signals.   So I mutated the amino acid residues in transmembrane domains and

checked their ability to make signals.
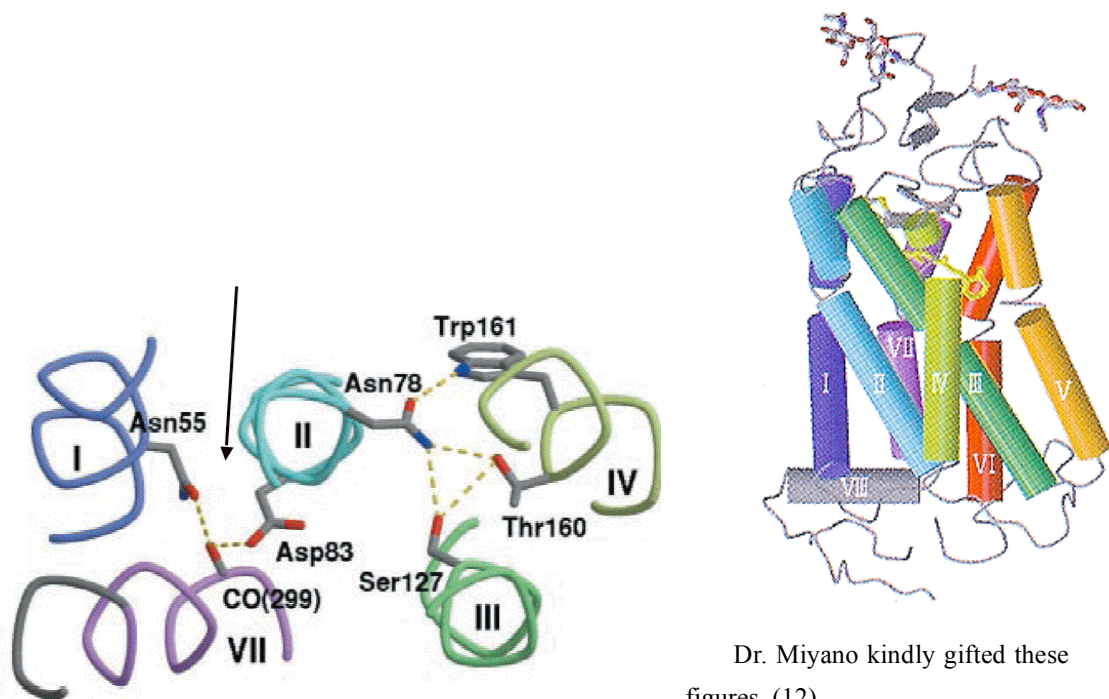
Dr. Miyano kindly gifted these figures. (12)

Left figure indicates hydrogen bond between asparagine in TM1, aspartic acid in TM2, and alanine in TM7 (arrow).

Right figure indicates structure of rhodopsin.

Recently, three-dimensional structural analyses of some GPCRs were reported (12-14), and many structural characters were disclosed. They made it possible to deduce the structure of other GPCRs based on their three-dimensional structural analyses. For example, asparagine in TM1, aspartic acid in TM2, and alanine in TM7 seem to make a hydrogen bond based on the crystal structure of rhodopsin. Since helices in a peptide chain tend to be bent at proline residues, it is likely that mutation of this residue caused a marked conformational change of the receptor. So, deficiency of these residues is likely to result in misfolding of the protein during its biosynthesis in the ER.

Proteins are synthesized in the ER and misfolded proteins are degraded by the ER-associated degradation (ERAD) system. Many ER chaperone proteins are working in this system and trying to make refolding of the misfolded proteins. But when they can not make refolding, misfolded proteins are retrotranslocated to the cytosol, conjugated ubiquitin by E1-E2-E3 ubiquitin ligase system and degraded by the proteasome (15). Some reports said that the different E3 ubiquitin ligase complex cooperatively works in case of recognizing the misfolding in different unfolded position (16, 17). But it is unclear the mechanism of recognition of the misfolded proteins in the ERAD system. At the start of this work, I hypothesized that mutant GPCRs accumulated in the ER and degraded by the ERAD system and tried to elucidate this mechanism.

Moreover, if mutated GPCRs are retained in the ER, it is difficult to elucidate the function of these receptors in living cells due to their aberrant folding. To address this question, I used pharmacological chaperones (18-20). Most of them are specific ligands of the receptors. They are able to rescue the intracellular retention of several misfolded proteins by stabilizing their conformation and/or enhancing refolding to pass through the ERAD system. Some of them are used to treat congenital diseases such as nephrogenic diabetes insipidus and hypogonadotropic hypogonadism which are caused by the mutations of vasopressin V2 receptor (V2R) and gonadotropin-releasing hormone receptor (GnRHR), respectively. I thought that structurally altered GPCRs retained in the ER might be exported to the cell surface by binding specific ligands, so I chose some specific ligands of the receptor.
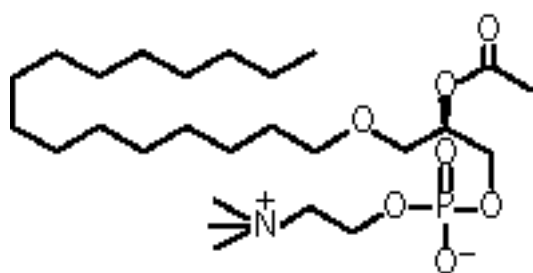
Structure of platelet-activating factor (PAF) (21)

To address the issue, PAF receptor was used as a model system. PAF is a biologically active phospholipids. It was initially focused for its potential to induce platelet aggregation and secretion, but many works have elucidated potent biological

actions of PAF in a broad range of cell types and tissues, many of which also produce

PAF.   Molecular cloning of PAFR was done in 1991 (22), and intracellular signal

transduction pathways, including turnover of phosphatidylinositol, elevation in

intracellular calcium concentration, and activation of MAPK have been underscored.

Generating PAFR mutant animals (PAFR overexpressing mice and PAFR deficient

mice) have revealed that PAF is related with airway responsiveness and acute lung

injury in Dr. Takao Shimizu's laboratory (23, 24).

In this report, I generated various PAFRs mutated at conserved residues in order to

demonstrate the importance of them for the correct folding of GPCRs during their

biosynthesis in the ER.   I successfully identified several residues that could be crucial

for correct folding by determining the deficiency in expression of these receptors at the

cell surface.   Although the binding properties of ligands to these mutants have been

previously examined using membrane preparations (3-7, 10), little is known on the

functional features of these mutants in living cells.   To address this issue, I detected

the expression of mutant PAFRs on the cell surface by treatment with PAF ligands as

pharmacological chaperones and carried out functional analysis.   Consequently, I

identified residues required for the export from the ER, and treatment with

pharmacological chaperones helps it pass the quality control machinery in the ER.

Experimental Procedures

Materials -

PAF and methylcarbamyl (mc)-PAF C-16 were purchased from Cayman Chemical (Ann Arbor, MI, USA).   Anti-haemagglutinin (HA) antibody (clone 3F10), Endoglycosidase H (Endo-H), and *N*-Glycosidase F (PNGase-F) were from Roche Applied Science (Penzberg, Germany); anti-Ub antibody (P4D1), horseradish peroxidase (HRP)-conjugate anti-rat IgG, and protein A/G plus-agarose were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and ECL Western Blotting Detection System, HRP-conjugated anti-mouse IgG whole antibody, and HRP-conjugate streptavidin (SA-HRP) were from GE Healthcare (Little Chalfont, UK). Phycoerythrin (PE)-conjugated anti-rat IgG was from Beckman Coulter (Fullerton, CA, USA).   Anti-Calreticulin antibody was from Stressgen Bioreagents (Ann Arbor, MI, USA).   Anti-Calnexin antibody was from BD Biosciences Pharmingen (San Jose, CA, USA).   Anti-golgin-97 antibody, Alexa 488-conjugated anti-rat IgG, Alexa 546-conjugated anti-mouse IgG, and Alexa 546-conjugated anti-rabbit IgG were from Invitrogen (Carlsbad, CA, USA).   Y-24180 was donated by Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan).   Anti-Flag M2 antibody was from Sigma-Aldrich (St. Louis, MO, USA).   MG-132 was from BIOMOL (Plymouth Meeting, PA, USA).

Construction of Mutant GPCRs -

Using N-terminally HA-tagged human PAFR (HA-hPAFR), human leukotriene B$_4$ type-2 receptor (HA-hBLT2), or human GPR43 (HA-hGPR43) as templates, mutant receptors were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. The primer sets utilized are listed below.

Primer sequences for generating mutants -

PAFR D63A Forward (5'-GAACCTCACCATGGCGGCAATGCTCTTCTTGATC-3')

PAFR D63A Reverse (5'-GATCAAGAAGAGCATTGCCGCCATGGTGAGGTTC-3')

PAFR P247A Forward (5'-CTGCTTCGTGGCCCACCACGTGGTG-3')

PAFR P247A Reverse (5'-CACCACGTGGTGGGCCACGAAGCAG-3')

PAFR P290A Forward

 (5'-CAACTGTGTCTTAGACGCTGTTATCTACTGTTTCC-3')

PAFR P290A Reverse

 (5'-GGAAACAGTAGATAACAGCGTCTAAGACACAGTTG-3')

PAFR C90A Forward (5'-CTCCCCAAATTCCTGGCCAACGTGGCTGGCTGC-3')

PAFR C90A Reverse (5'-GCAGCCAGCCACGTTGGCCAGGAATTTGGGGAG-3')

PAFR L59A Forward (5'-ATGGTGAACGCCACCATGGCG-3')

PAFR L59A Reverse (5'-CGCCATGGTGGCGTTCACCATG-3')

PAFR M64A Forward (5'-ATGGCGGACGCCCTCTTCTTG-3')

PAFR M64A Reverse (5'-CAAGAAGAGGGCGTCCGCCATG-3')

PAFR F241A Forward (5'-TTGGCGGTGGCCATCATCTGC-3')

PAFR F241A Reverse (5'-GCAGATGATGGCCACCGCCAAG-3')

PAFR C244A Forward (5'-TTCATCATCGCCTTCGTGCCC-3')

PAFR C244A Reverse (5'-GGGCACGAAGGCGATGATGAAC-3')

PAFR F245A Forward (5'-ATCATCTGCGCCGTGCCCCAC-3')

PAFR F245A Reverse (5'-GTGGGGCACGGCGCAGATGATG-3')

PAFR D289A Forward (5'-TGTGTCTTAGCCCCTGTTATC-3')

PAFR D289A Reverse (5'-GATAACAGGGGCTAAGACACAG-3')

PAFR Y293A Forward (5'-CCTGTTATCGCCTGTTTCCTC-3')

PAFR Y293A Reverse (5'-GAGGAAACAGGCGATAACAG-3')

BLT2 D68A Forward (5'- CCTGGCGCTGGCCGCCGGCGCGGTGCTGC-3')

BLT2 D68A Reverse (5'- GCAGCACCGCGCCGGCGGCCAGCGCCAGG-3')

BLT2 P239A Forward (5'- GCTCTGGGCCGCCTACCACGCAGTC-3')

BLT2 P239A Reverse (5'- GACTGCGTGGTAGGCGGCCCAGAGC-3')

GPR43 D55A Forward (5'-ACGCTGGCCGCCCTCCTCCTG-3')

GPR43 D55A Reverse (5'-AGGTGGAGGGCGGCCAGCGTC-3')

GPR43 P237A Forward (5'-TGCTTCGGAGCTTACAACGTG-3')

GPR43 P237A Reverse (5'-ACGTTGTAAGCTCCGAAGCAG-3')

Construction of Mutant GPCRs -

cDNA of hHRD1 (human HMG-CoA reductase degradation protein 1) was obtained from Invitrogen.   Flag tag was added to the C-terminus by PCR.

Cell Culture and Transfection -

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were transfected with a wild-type (WT) or mutated receptor-expressing plasmid using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. A stable HeLa cell line expressing the *Tet* repressor (HeLa Tet-On Cell Line) was from Clontech (Palo Alto, CA) (25, 26).   Stable cell lines with inducible expression of WT or mutant PAFRs were established by transfecting the pTRE plasmid bearing the appropriate PAFRs into the HeLa Tet-On Cell Line with the Lipofectamine 2000 transfection reagent under geneticin (1 mg/ml; Invitrogen) and hygromycin (100 μg/ml; Wako, Osaka, Japan) selections.   Cells grown in the presence of geneticin and hygromycin were isolated, expanded, and then tested for doxycycline (Dox) (1 μg/ml; Clontech)-inducible expression of the appropriate recombinant proteins by Western blotting (Fig. 1).   The clones used for experiments showed very low basal receptor
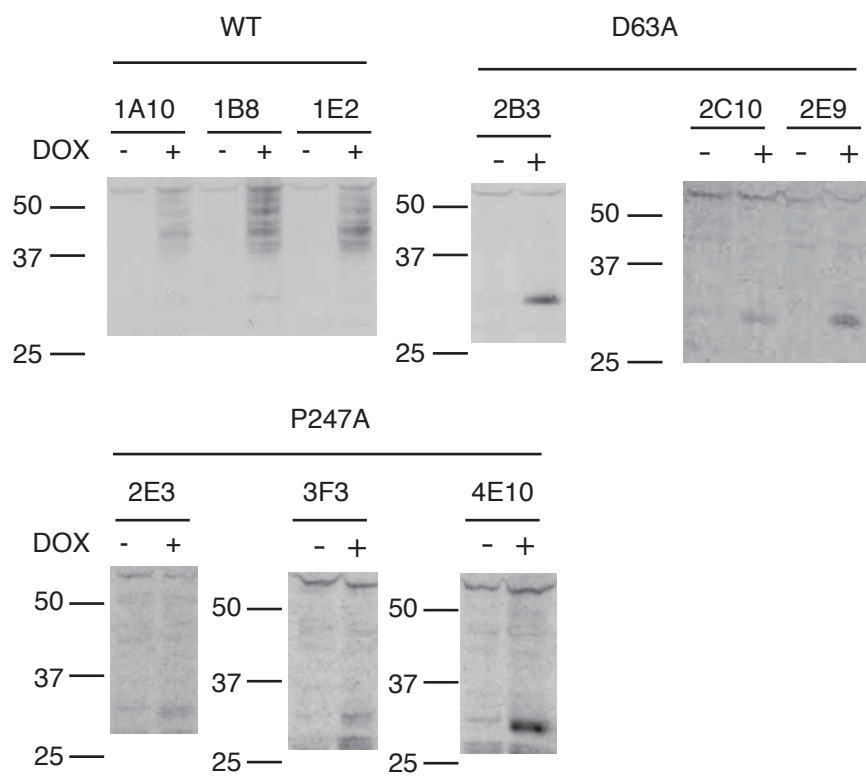
FIGURE 1.

Figure 1.   Establishment of HeLa cell lines in Dox-inducible manner expressing WT or mutant PAFRs.

Dox-induced expression of WT and mutant PAFRs in the established cell lines HeLa-WT, HeLa-D63A, and HeLa-P247A, which inductively produce the receptors by addition of Dox. The expressions of each receptor were evaluated by Western blotting using anti-HA antibody. The molecular standards are shown at the left. Data are representative of three independent experiments with identical results.

expression.   Cells were plated and cultured for 16 h, and receptor expression was induced by adding Dox (1 μg/ml) in the culture medium for 24 h.

Flow Cytometry -

Intact cells were incubated with anti-HA antibody (3F10; 2 μg/ml) in phosphate-buffered saline (PBS) containing 2% goat serum at room temperature for 30 min, followed by staining with PE-conjugated anti-rat IgG at room temperature for 30 min.   EPICS XL (Beckman Coulter) was used for flow cytometry.

Western Blotting -

Two days after transfection, cells were harvested with PBS containing 2 mM EDTA. Cells were disrupted by sonication in ice-cold sonication buffer (25 mM HEPES-NaOH, pH 7.4, 0.25 M sucrose, 10 mM $MgCl_2$) plus protease inhibitor mixture (Roche, one tablet in 50 ml).   The cell debris was removed by centrifugation at 800 x g for 5 min at 4°C, and the resultant supernatants were used as protein samples.   The protein concentration was determined with the Bradford method (27) using a Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard.   For Western blot analysis, protein samples were separated on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and transferred to a nitrocellulose membrane.   After blocking with 5% skim milk in TBS-T (20 mM Tris-buffered saline, pH 7.4, 0.1%

Tween 20), blots were probed with the primary antibody for 1 h.   The membrane was washed with TBS-T and incubated with biotin-conjugated antibody or HRP-conjugated antibody for 1 h.   In the case of the biotin-conjugated antibody, the membrane was incubated with SA-HRP (GE Healthcare) for 0.5 h.   Signal was visualized using an ECL Western Blotting Detection System (GE healthcare).

Endoglycosidase Treatment of PAFRs -

Protein samples were obtained by the methods described above.   Protein samples were treated with Endo-H (0.005 units) in 50 µl of buffer (11.7 nM Na_2HPO_4, 168.3 nM NaH_2PO_4, 0.4% SDS, 20 mM EDTA, 2% 2-mercaptethanol) or treated with PNGase-F (1 unit) in 50 µl of buffer (139.2 nM Na_2HPO_4, 40.8 nM NaH_2PO_4, 0.4% SDS, 20 mM EDTA, 2% 2-mercaptethanol) for 16 h at 4°C (28, 29).   The resultant protein samples were suspended in sampling buffer (25 mM Tris-HCl, pH 6.5, 5% glycerol, 1% SDS, and 0.05% bromphenol blue) and were subjected to Western blot analysis.

Immunoprecipitation -

All experements were carried out at 4°C.   Cells were lysed with lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40 [NP-40, nacalai tesque, Kyoto, Japan], 0.5% Sodium deoxycholate, protease inhibitor mixture [one tablet in 25 ml]).   Total cell lysates were subjected to incubation with anti-HA antibody (3F10) and protein

A/G plus-agarose for 16 h.   After centrifugation at 10,000 x *g* for 10 min, the pellets

were washed with wash buffer-1 (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% NP-40,

0.05% sodium deoxycholate) and then with wash buffer-2 (50 mM Tris-HCl pH 7.5,

0.1% NP-40, 0.05% sodium deoxycholate).   The resultant pellets were suspended in

sampling buffer and subjected to Western blot analysis.   In case of co-transfection of

HA-hPAFR and Flag-hHRD1, Western blot analysis was undergone by anti-Flag M2

antibody.

Immunofluorescence Confocal Microscopy Analysis -

Cells (5 x 10^5) were seeded into collagen-coated glass-bottomed 35-mm dishes

(MatTek Corporation, Ashland, MA).   After incubation for 24 h at 37°C, the cells were

fixed with 2% paraformaldehyde for 10 min at room temperature and rinsed twice with

ice-cold PBS.   Subsequently, the cells were incubated with 1/4x permeabilization

reagent (Beckman Coulter) for 10 min at room temperature.   Then, primary antibodies

(anti-HA [1:100, 3F10] and anti-Calreticulin [1:200] (30) or anti-golgin-97 [1:50] (31))

were added and incubated for 2 h.   After washing the cells with ice-cold PBS,

secondary antibodies (Alexa 488-conjugated anti-rat IgG [1:200] and Alexa

546-conjugated anti-rabbit IgG [1:200] or Alexa 546-conjugated anti-mouse IgG

[1:200]) were added and incubated for 2 h.   Images were obtained using an LSM510

Laser Scanning Confocal Microscope (Carl Zeiss, Jena, Germany) equipped with an argon laser.

Calcium Mobilization Assay -

HeLa cells transiently transfected with WT HA-PAFR, D63A, P247A, or empty vector were applied to a 96-well plate (4 x 10^4 cells/well) and incubated for 16 h. Then, Y-24180 (1 µM) was added to the culture medium and incubated for 24 h at 37°C. The cells were incubated with loading buffer (buffer A [1x HBSS and 2.5 mM probenecid]) (Sigma-Aldrich), and 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01% BSA]) containing 4 µM Fluo 3-AM (Dojindo, Kumamoto, Japan), 0.04% Pluronic acid (Molecular Probes), at 37°C for 1 h. Then, they were washed twice with buffer A. Intracellular Ca^{2+} mobilization was monitored with a scanning fluorometer (FLEX station, Molecular Devices Corp.) (32, 33). Emission fluorescence at 525 nm in response to excitation at 485 nm was measured. The relative fluorescence units (RFU, Max – Min) were indicated.

Statistical Analysis -

Data were analyzed for statistical significance using Prism 4 software (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at $p < 0.05$, 0.01 as indicated.

Results

Requirement of Conserved Residues for Cell Surface Expression of PAFR -

In the transmembrane domains of the rhodopsin-type GPCRs, several amino acid residues are highly conserved, *e.g.*, aspartic acid and leucines in TM2; phenylalanine, cysteine, tryptophan and proline in TM6; and asparagine/aspartic acid, proline and tyrosine in TM7 (Fig. 2).   I tried to elucidate the influence of changing these residues. So I generated alanine-substituted mutants in these conserved residues of HA-tagged human PAFR (HA-hPAFR) (Fig. 3*A* and *B*) and analyzed cell surface expression of these mutants semiquantitatively by flow cytometry in transiently transfected HeLa cells.   As shown in Fig. 4*A* and *B*, many of these mutant HA-hPAFRs, *e.g.*, L59A and D63A in TM2, F245A and P247A in TM6, and D289A and P290A in TM7, showed a marked decrease in cell surface expression.   Quantities of HA-hPAFRs were almost the same in Western Blotting as shown in Fig. 4*C*.   In particular, mutations of the aspartic acid in TM2 (D63) and the proline in TM6 (P247) resulted in drastic impairment of the cell surface expression.   The aspartic acid in TM2 make a hydrogen bond with asparagine in TM1 and alanine in TM7, and helices in a peptide chain tend to be bent at proline residues based on structural analysis of rhodopsin (12), so I chose these two residues to go to next step.

FIGURE 2.

TM2

```
Rho      HKKLRTPLNYILLNLAVADLFMVLGGFTSTLYTSLHG
PAFR     PCKKFNEIKIFMVNLTMADMLFLITLPLWIVYYQNQG
BLT2     PARGRPLAATLVLHLALADGAVLLLTPLFVAFLTRQA
GPR43    RQPQPAPVHILLLSLTLADLLLLLLLPFKIIEAASNF
TDAG8    QPKKESELGIYLFSLSLSDLLYALTLPLWIDYTWNKD
S1P1     TKKFHRPMYYFIGNLALSDLLAGVAYTANLLLSGATT
CB1      RSLRCRPSYHFIGSLAVADLLGSVIFVYSFIDFHVFH
IL8      SRVGRSVTDVYLLNLALADLLFALTLPIWAASKVNGW
H1       ERKLHTVGNLYIVSLSVADLIVGAVVMPMNILYLLMS
5HT-1A   ERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLN
NPY-Y1   QKEMRNVTNILIVNLSFSDLLVAIMCLPFTFVYTLMD
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TM6

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Rho      KAEKEVTRMVIIMVIAFLICWVPYASVAFYIFTHQGS
PAFR     EVKRRALWMVCTVLAVFIICFVPHHVVQLPWTLAELG
BLT2     RHGARVGRLVSAIVLAFGLLWAPYHAVNLLQAVAALA
GPR43    QRRRRAVGLAVVTLLNFLVCFGPYNVSHLVGYHQRKS
TDAG8    KEKKRIIKLLVSITVTFVLCFTPFHVMLLIRCILEHA
S1P1     EKSLALLKTVIIVLSVFIACWAPLFILLLLDVGCKVK
CB1      RMDIRLAKTLVLILVVLIICWGPLLAIMVYDVFGKMN
IL8      GQKHRAMRVIFAVVLIFLLCWLPYNLVLLADTLMRTQ
H1       NRERKAAKQLGFIMAAFILCWIPYFIFFMVIAFCKNC
5HT-1A   ARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESS
NPY-Y1   SETKRINIMLLSIVVAFAVCWLPLTIFNTVFDWNHQI
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TM7

```
Rho      SNFGPIFMTIPAFFAKSAAIYNPVIYIMMNKQFRNCM
PAFR     HQAINDAHQVTLCLLSTNCVLDPVIYCFLTKKFRKHL
BLT2     GGAGQAARAGTTALAFFSSSVNPVLYVFTAGDLLPRA
GPR43    QRKSPWWRSIAVVFSSLNASIDPLLFYFSSSVVRRAF
TDAG8    GKRTYTMYRITVALTSLNCVADPILYCFVTETGRYDM
S1P1     TCDILFRAEYFLVLAVLNSGTNPIIYTLTNKEMRRAF
CB1      NKLIKTVFAFCSMLCLLNSTVNPIIYALRSKDLRHAF
IL8      RNNIGRALDATEILGFLHSCLNPIIYAFIGQNFRHGF
H1       NCCNEHLHMFTIWLGYINSTLNPLIYPLCNENFKKTF
5HT-1A   CHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAF
NPY-Y1   TCNHNLLFLLCHLTAMISTCVNPIFYGFLNKNFQRDL
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Figure 2.   Conse rved amino acid residues in TMs of rhodopsin-type GPCRs.

Conserved residues in TM2, TM6, and TM7 of several rhodopsin-type GPCRs are shown in *red*. Transmembrane domains are *underlined*. *Rho*, human rhodopsin; *S1P1*, human sphingosine-1-phosphate type-1 receptor; *CB1*, human cannabinoid type-1 receptor; *IL8*, human interleukin-8 receptor; *H1*, human histamine type-1 receptor; *5HT-1A*, human serotonin type-1A receptor; *NPY-Y1*, human neuropeptide-Y Y1 receptor.
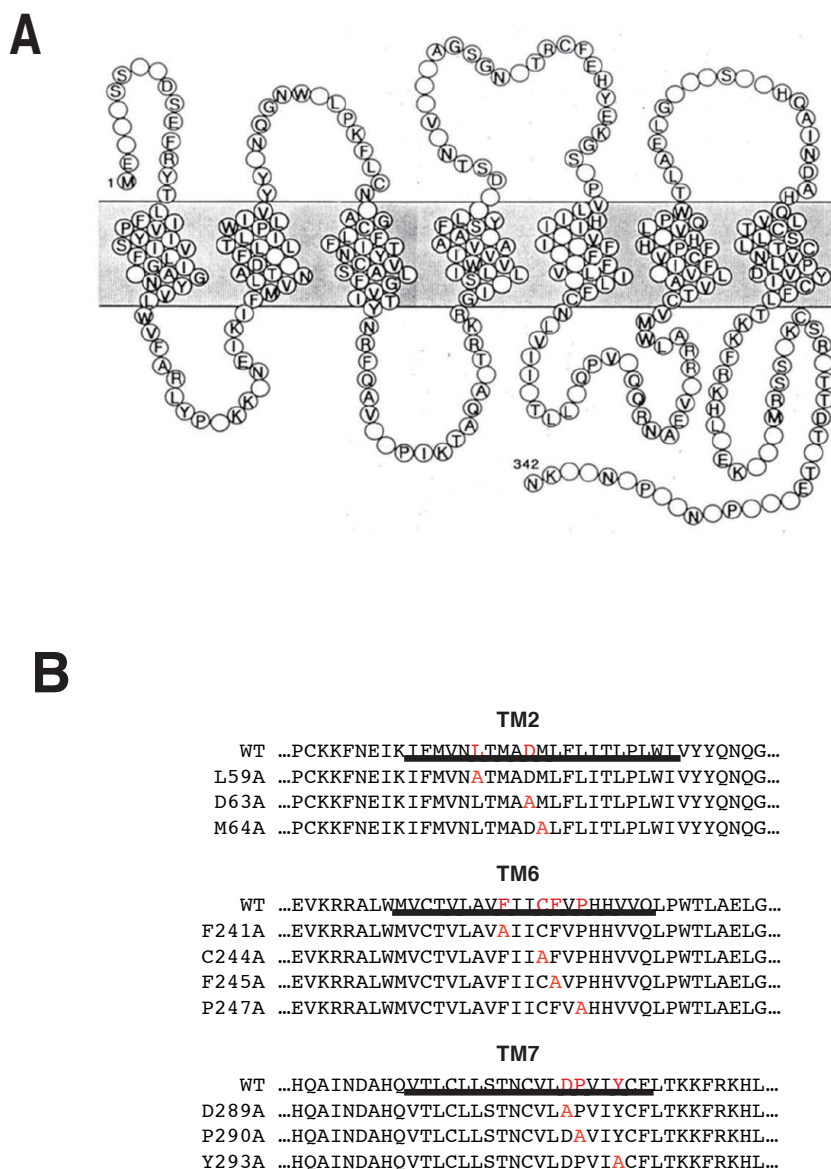
FIGURE 3

A

B

TM2

```
      WT ...PCKKFNEIKIFMVNLTMADMLFLITLPLWIVYYQNQG...
    L59A ...PCKKFNEIKIFMVNATMADMLFLITLPLWIVYYQNQG...
    D63A ...PCKKFNEIKIFMVNLTMAAMLFLITLPLWIVYYQNQG...
    M64A ...PCKKFNEIKIFMVNLTMADALFLITLPLWIVYYQNQG...
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TM6

```
      WT ...EVKRRALWMVCTVLAVFIICFVPHHVVQLPWTLAELG...
   F241A ...EVKRRALWMVCTVLAVAIICFVPHHVVQLPWTLAELG...
   C244A ...EVKRRALWMVCTVLAVFIIAFVPHHVVQLPWTLAELG...
   F245A ...EVKRRALWMVCTVLAVFIICAVPHHVVQLPWTLAELG...
   P247A ...EVKRRALWMVCTVLAVFIICFVAHHVVQLPWTLAELG...
```

TM7

```
      WT ...HQAINDAHQVTLCLLSTNCVLDPVIYCFLTKKFRKHL...
   D289A ...HQAINDAHQVTLCLLSTNCVLAPVIYCFLTKKFRKHL...
   P290A ...HQAINDAHQVTLCLLSTNCVLDAVIYCFLTKKFRKHL...
   Y293A ...HQAINDAHQVTLCLLSTNCVLDPVIACFLTKKFRKHL...
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Figure 3.   Mutated amino acid residues in TM domains.

   A : A putative transmembrane structure of the hPAFR (3).

   B : Ten mutant HA-hPAFRs generated in this study.   Conserved residues and substituted alanines in each mutant PAFR are indicated in *red*.   Transmembrane domains are *underlined*.

FIGURE 4.

A

B
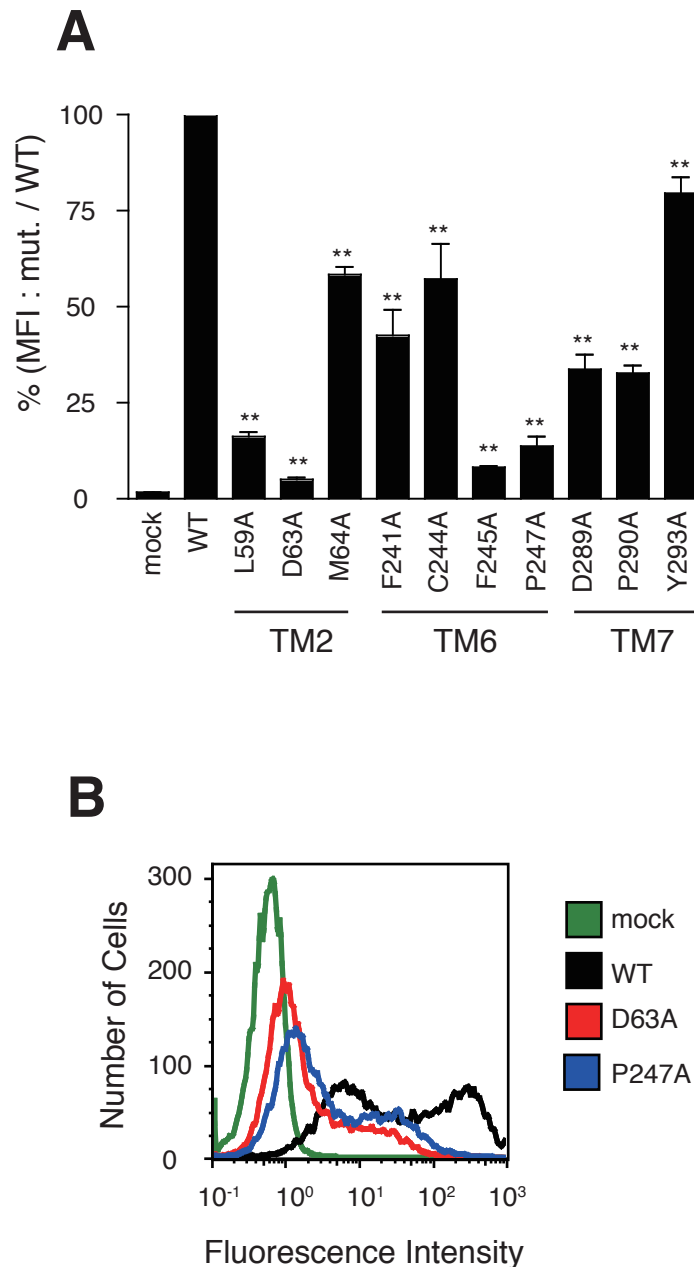
Figure 4.   Requirement of conserved residues in rhodopsin-type GPCRs for the cell surface expression of HA-hPAFR.

  A : The cell surface expression levels of each mutant HA-hPAFR in HeLa cells were determined by flow cytometric analysis.   After staining with anti-HA and PE-conjugated anti-rat IgG as primary and secondary antibodies, respectively, the fluorescence intensity of each transfectant was measured.   The expression levels were evaluated by mean fluorescence intensities (MFI), represented as a percentage of WT HA-hPAFR.   Data are represented as means + standard deviations (S.D.) (n = 3). Statistical significance was analyzed using ANOVA with Dunnett *post hoc* pairwise comparisons   **, $p < 0.01$ (versus WT).

  B : One of the results from the flow cytometric analysis is shown.   HeLa cells were transiently transfected with WT HA-hPAFR, D63A, P247A, or empty vector.

FIGURE 4.

C
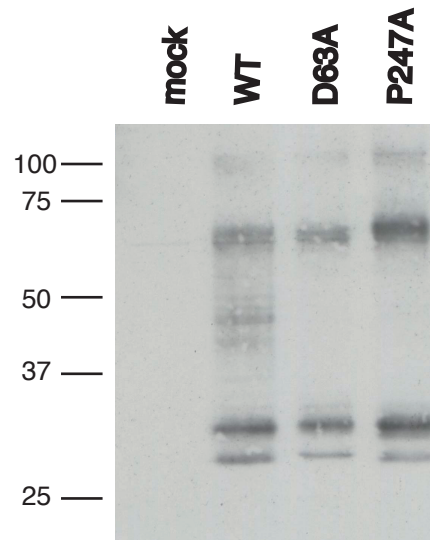
IB : anti-HA (3F10)

C : One of the results of Western Blotting is shown. HeLa cells were transiently transfected with WT HA-hPAFR, D63A, P247A, or empty vector.

Deficiency of Cell Surface Expression of Other GPCRs by Mutating Crucial Residues -

To gain further evidence for the influence of changing the conserved residues in TM2 and TM6 for GPCR trafficking, I constructed other mutant GPCRs using hBLT2 (34) and hGPR43 (35), which introduced mutations into positions corresponding to D63 and P247 in HA-hPAFR.   Similar to the results obtained with HA-hPAFR, the cell surface expression profiles of these mutant receptors were substantially reduced (Fig. 5), suggesting that these residues could play a pivotal role in the cell surface expression of the rhodopsin-type GPCRs.

Accumulation of Mutant HA-hPAFRs in the ER -

To dissect the deficiency in cell surface expression of the mutant HA-hPAFRs, I investigated the subcellular localization of these receptors in HeLa cells using confocal immunolocalization analysis.   As shown in Fig. 6*A*, I found that WT HA-hPAFR was expressed mainly on the plasma membrane 48 h after transfection of HeLa cells.   In contrast, D63A and P247A mutants exhibited marked localization to the perinuclear regions of the cells, resulting in complete deficiency of their cell surface expression. There was no merged signal between the mutant HA-hPAFRs and golgin-97 (31), a Golgi apparatus marker, although the mutant HA-hPAFRs did overlap extensively with the ER marker calreticulin (30).   The accumulation of the D63A and P247A mutants in
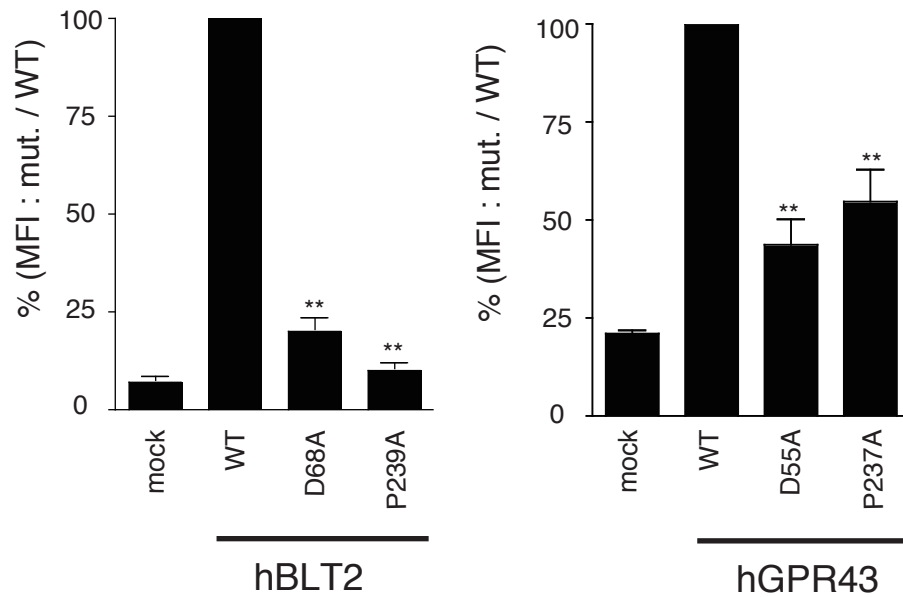
FIGURE 5.

Figure 5.   Deficiency of cell surface expression by mutation of the pivotal residues in HA-hBLT2 and HA-hGPR43.

    WT and two mutant receptors, with point mutations in the aspartic acid in TM2 or the proline in TM6, were prepared using HA-hBLT2 (*left*) and HA-hGPR43 (*right*). The cell surface expression levels of each GPCR were determined by flow cytometric analysis.   After staining with anti-HA and PE-conjugated anti-rat IgG as primary and secondary antibodies, respectively, the fluorescence intensity of each transfectant was measured.   The expression levels were evaluated by MFI, represented as a percentage of the WT receptor.   Data are represented as means + S.D. (n = 3).   Statistical significance was analyzed using ANOVA with Dunnett *post hoc* pairwise comparisons **, $p < 0.01$ (versus WT).

FIGURE 6.

A
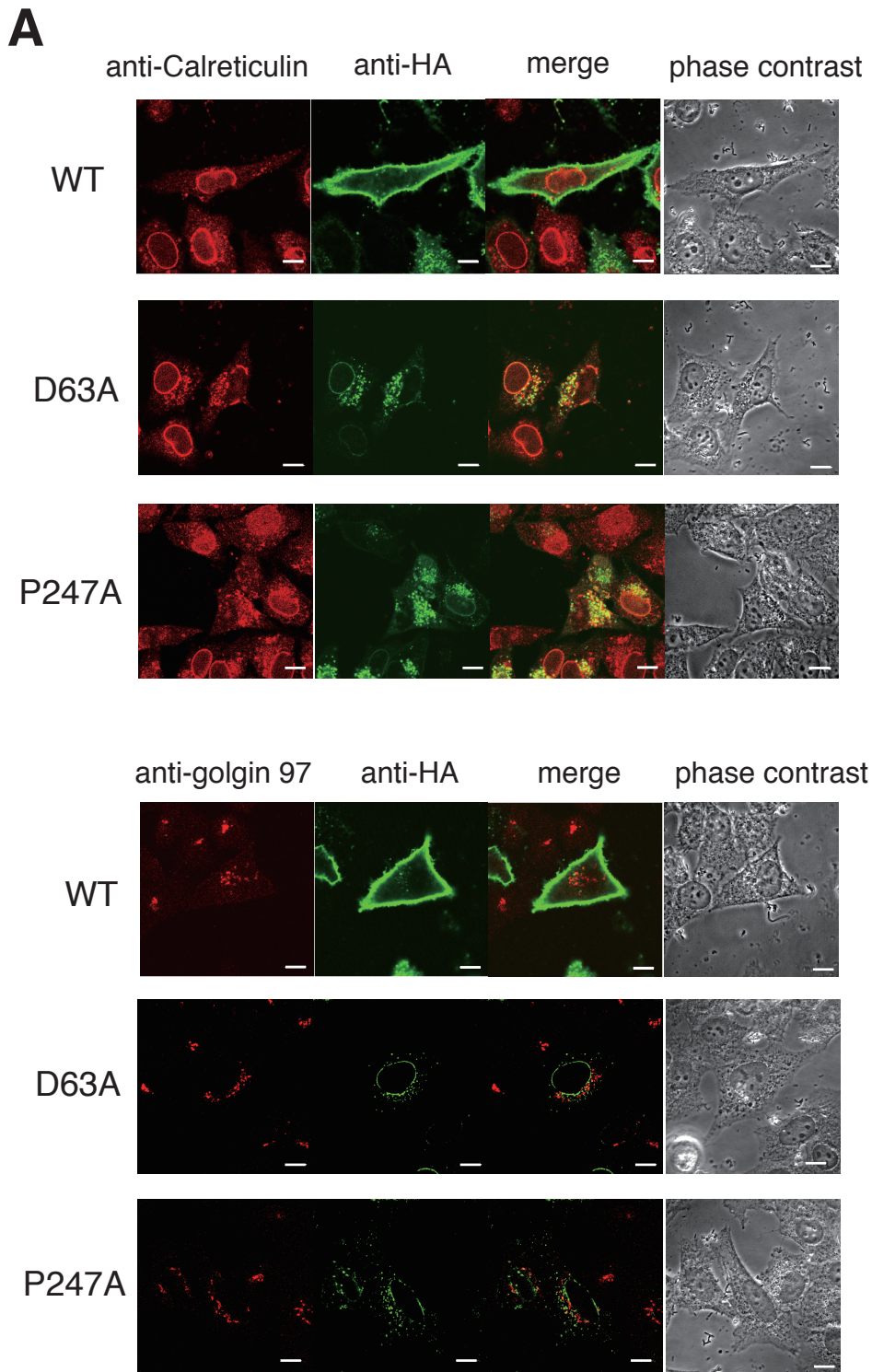
Figure 6. Accumulation of mutant HA-hPAFRs in the ER.

A : Subcellular localizations of WT and mutant HA-hPAFR were analyzed by immunofluorescence confocal microscopy. HeLa cells were transfected with WT HA-hPAFR, D63A, or P247A and subjected to immunocytochemical analysis 48 h post-transfection. Calreticulin, golgin-97, and HA-tagged hPAFRs were visualized using anti-Calreticulin (*upper, red*), anti-golgin-97 (*lower, red*), and anti-HA (*green*) antibodies, respectively. White bars in the photos indicate 10 μm. The data are representative of three independent experiments with identical results.

the ER was further confirmed by examining the glycosylation of the receptors, which is linked and further modified during trafficking to the cell surface (28, 29). With respect to WT HA-hPAFR, several molecular masses of the protein - a broad species (approx. 40-50 kDa), 30 kDa, and 27 kDa - were detected by Western blot analysis (Fig. 6*B*). Because the broad species disappeared following treatment with PNGase-F that cleaves all types of *N*-glycans, but not with Endo-H that specifically digests high mannose glycans, these proteins could be fully glycosylated (probably complex glycosylated types). On the other hand, the 30 kDa band was shifted to 27 kDa by the treatments with PNGase-F and Endo-H, suggesting that the 30 kDa and 27 kDa bands corresponded to a core conjugated glyco-chain and a non-glycosylated forms of the protein, respectively, probably localized in the ER. As was previously observed (36), the detected proteins ran faster than their predicted molecular masses (approx. 39 kDa for non-glycosylated hPAFR). In contrast, the broad species were not detected in the D63A or P247A mutant cells, although similar shifts of the core chain conjugated band to the non-glycosylated form were observed by treatments with PNGase-F and Endo-H. It is likely that the 30 kDa band, especially in the D63A mutant, probably contains modification(s) resistant to glycosidases, because the band did not completely disappear upon treatment with these enzymes. Taken together, these results imply that both the D63A and P247A mutants accumulate in the ER.

FIGURE 6.

B
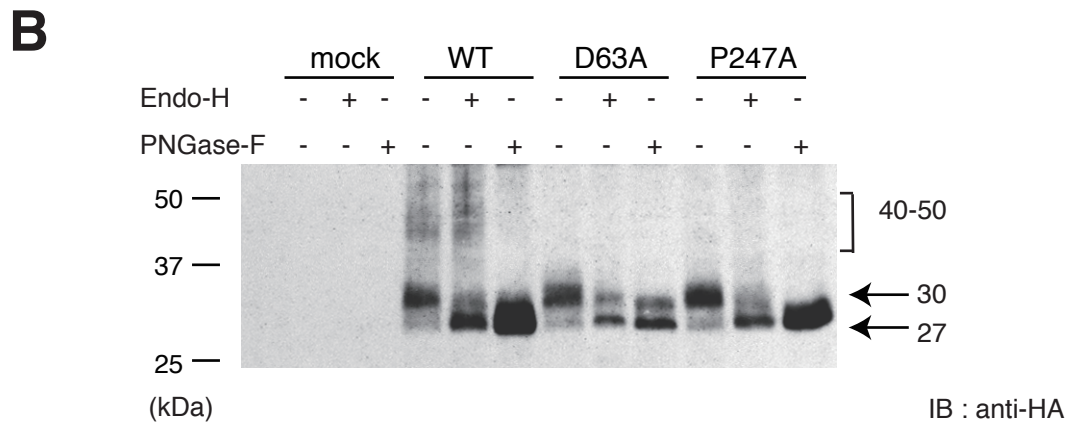
B : Protein preparations from cells transfected with WT HA-hPAFR, D63A, P247A, or empty vector were treated with Endo-H (0.005 units) or PNGase-F (1 unit). The results were analyzed by Western blotting using anti-HA antibody. Approximate molecular sizes are shown in *kDa* at the *right* with the standard proteins at the *left*. The data are representative of three independent experiments with identical results.

hHRD1 (E3 ubiquitin ligase) Works for Degradation of HA-hPAFR -

To know how mutant PAFRs degraded by the ERAD system, I examined what kind of ubiquitin E3 ligase works.   It has been reported that several factors implicate in the ERAD system in the ER (Fig. 7*A*).   For example, proteins unfolded in transmembrane domains are ubiquitinated by the E3 ubiquitin ligase complex containing hHRD1. Therefore, I co-transfected HA-hPAFR (WT, mutants, and vector only) and Flag-hHRD1 (WT and vector only), and these products were immunoprecipitated using anti-HA antibody, followed by immunoblotting by anti-Flag M2 antibody.   WT Flag-hHRD1 was co-immunoprecipitated with WT HA-hPAFR, D63A, and P247A (Fig. 7*B left*).   But I could not detect the denser band in mutant HA-hPAFRs than WT.   I could not see difference in the amount of the precipitated Flag-hHRD1 between WT and mutants (Fig. 7*B right*).

Action of Specific Ligands as Pharmacological Chaperones -

Recently, a class of compounds called pharmacological chaperones was shown to rescue the intracellular retention of several misfolded proteins by stabilizing their conformation and/or enhancing refolding (37).   To test whether specific ligands for PAFR act as pharmacological chaperones for the mutants, I examined the effects of two PAFR ligands, mc-PAF (38), an agonist of PAFR, and an antagonist Y-24180 (39, 40). I checked the export of PAFR from the ER by flow cytometry of transiently transfected
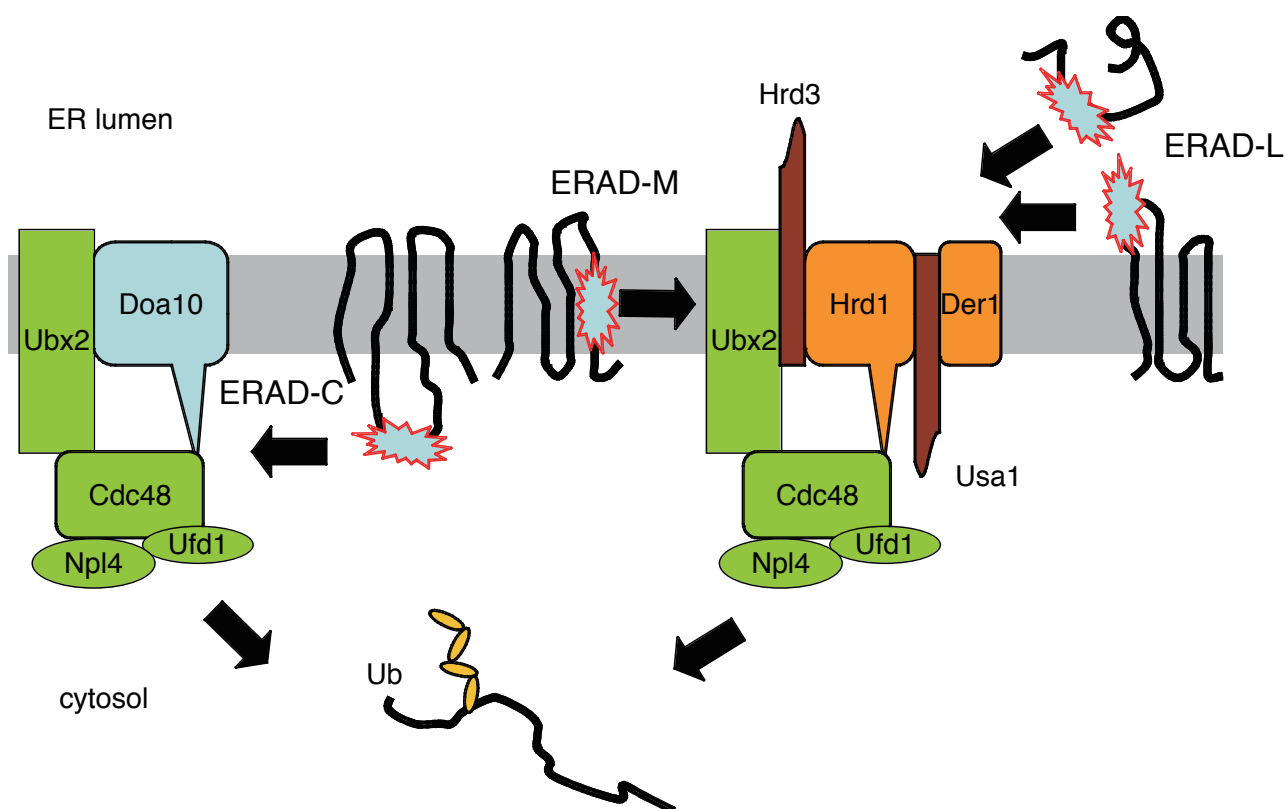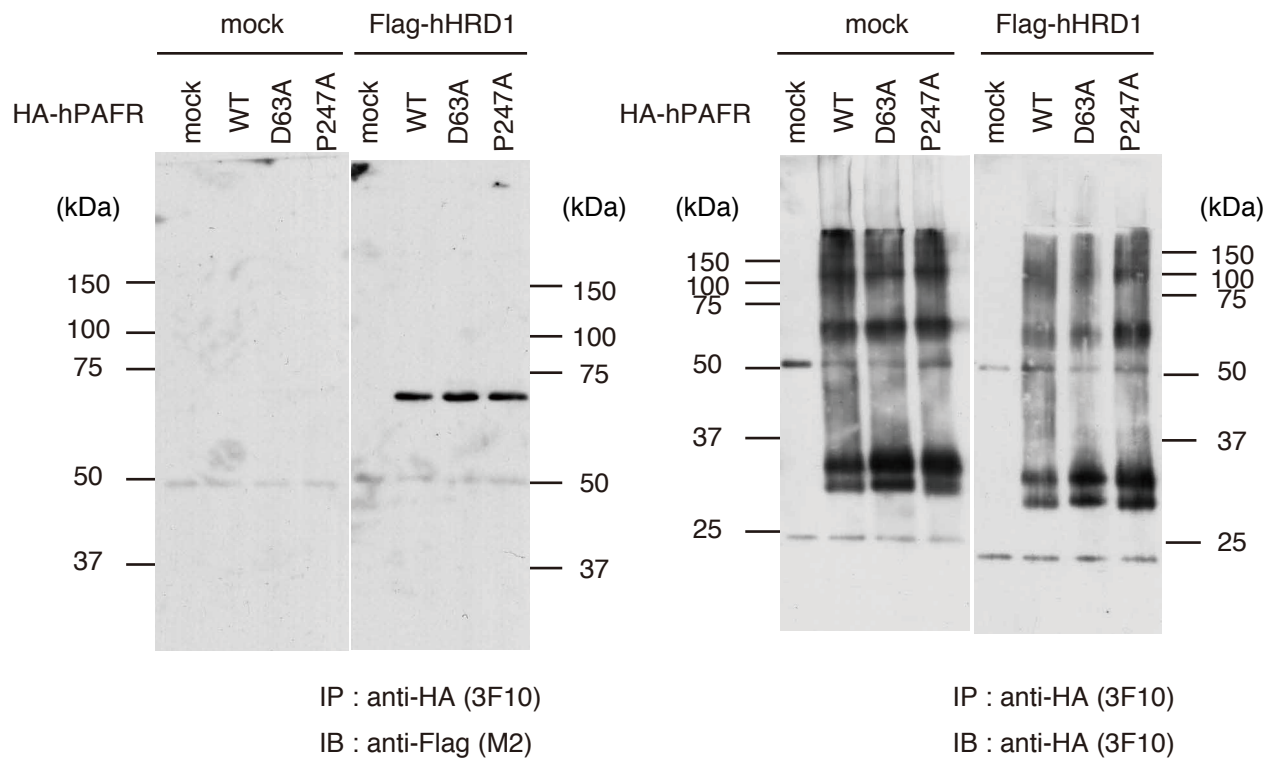
FIGURE 7.

A

**Figure 7.   hHRD1 (E3 ubiquitin ligase) plays a crucial role for degradation of HA-hPAFR.**

A : E3 ubiquitin ligase complexes involved in the ERAD-L, -M, and -C pathways. Stars show the location of the misfolded domain of a substrate.   Proteins unfolded in transmembrane domains or ER   lumen domains are ubiquitinated by complex containing hHRD1, whereas proteins unfolded in cytosol domains by complex containing hDoa10.

FIGURE 7.

B

B : Interaction of HA-hPAFRs and Flag-hHRD1. Flag-hHRD1 was transiently transfected into HeLa cells along with various PAFRs indicated in this figure. Before preparation of the proteins, these cells were incubated with 40 μM of MG-132 for 24 h. After immunoprecipitation of the HA-hPAFRs with anti-HA antibody, the precipitated samples were subjected to Western blot analysis using anti-Flag M2 (*left*, 8% polyacrylamide gel) and anti-HA (*right*, 10% polyacrylamide gel) antibodies. The molecular standards are shown. The data are representative of three independent experiments with identical results.

HeLa cells.   As shown in Fig. 8, the cell surface expression of the ER-accumulated mutant PAFRs, *e.g.*, L59A, D63A, and D289A, was markedly increased by addition of these ligands in the culture medium, except for that of a disulfide bond-disrupted type of PAFR mutant (C90A).   Interestingly, the cell surface expression of several mutants, *e.g.*, F245A, P247A, and P290A, was not rescued by mc-PAF.   Similar results were obtained using other HeLa cell lines, HeLa-D63A and HeLa-P247A, which inductively express the D63A and P247A mutants by the addition of Dox in the culture medium. Pharmacological chaperones probably got into the ER and bound with mutant receptors and made conformational change.   Differences of cell surface expression level of some mutants by each pharmacological chaperone may be because of its affinity to the receptors or its permeability of cell membrane.   Whether its pharmacological chaperone is agonist or antagonist (make signaling and receptor internalization) may also influence.   The chaperone activities of Y-24180 for these mutants were dose- and time-dependent (Fig. 9*A* and *B*).   The compound exhibited effects at 10-100 nM (Fig. 9*A*).   These mutants appeared on the cell surface 30 min after adding the ligand, and maximal expression was obtained at 6 h (Fig. 9*B*).

Promotion of Export of the Mutated PAFRs from the ER by the Pharmacological Chaperone -

FIGURE 8.
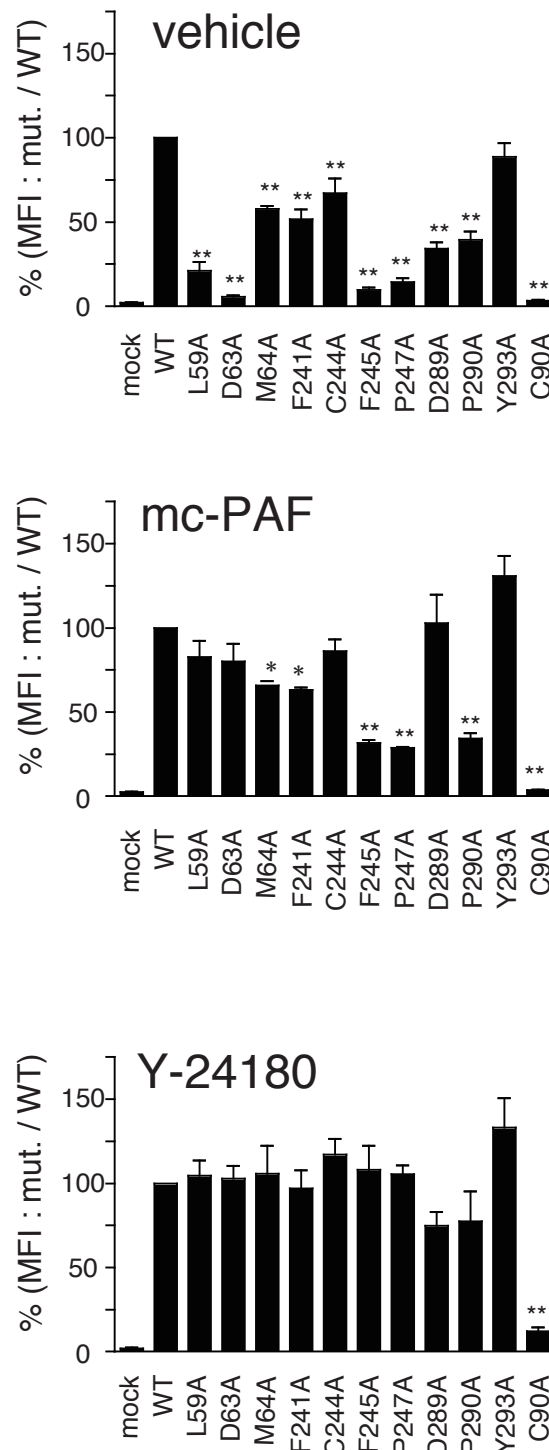
Figure 8.   Action of specific ligands as pharmacological chaperones.

Twenty-four hours after transfection of the various mutant HA-hPAFRs into HeLa cells, these cells were treated with or without 1 μM mc-PAF or 1 μM Y-24180 for 24 h at 37°C.   The cell surface expression levels of each mutant HA-hPAFR were determined by flow cytometric analysis.   After staining with anti-HA and PE-conjugated anti-rat IgG as primary and secondary antibodies, respectively, the fluorescence intensity of each transfectant was measured.   The expression levels were evaluated by MFI, represented as a percentage of WT HA-hPAFR.   Data are represented as means + S.D. (n = 3).   Statistical significance was analyzed using ANOVA with Dunnett *post hoc* pairwise comparisons   *, p<0.05; **, *p* < 0.01 (versus WT).

FIGURE 9.

A
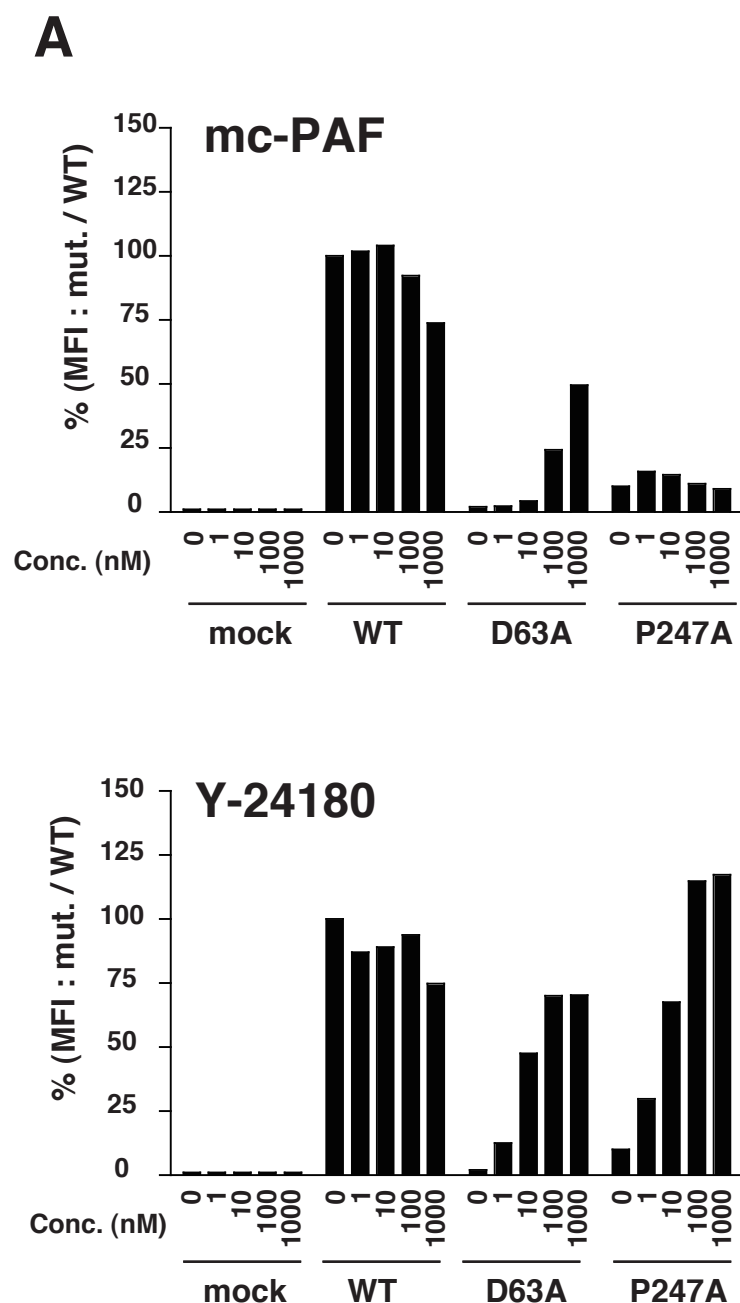
Figure 9. Dose- and time- dependencies of the effects of pharmacological chaperones.

A : Dose-dependent effects of mc-PAF (*left*) and Y-24180 (*right*) on the cell surface expression of the mutant HA-hPAFRs. These experiments were carried out using the cell lines HeLa-WT, HeLa-D63A, HeLa-P247A, and HeLa-mock cells, which inductively produce the receptors by addition of Dox. Twenty-four hours after the addition of Dox, the cells were treated with the indicated concentrations of mc-PAF or Y-24180 for 24 h. The expression levels were evaluated by MFI, represented as a percentage of WT HA-hPAFR at 0 nM compound. The data are representative of three independent experiments with identical results.

FIGURE 9.
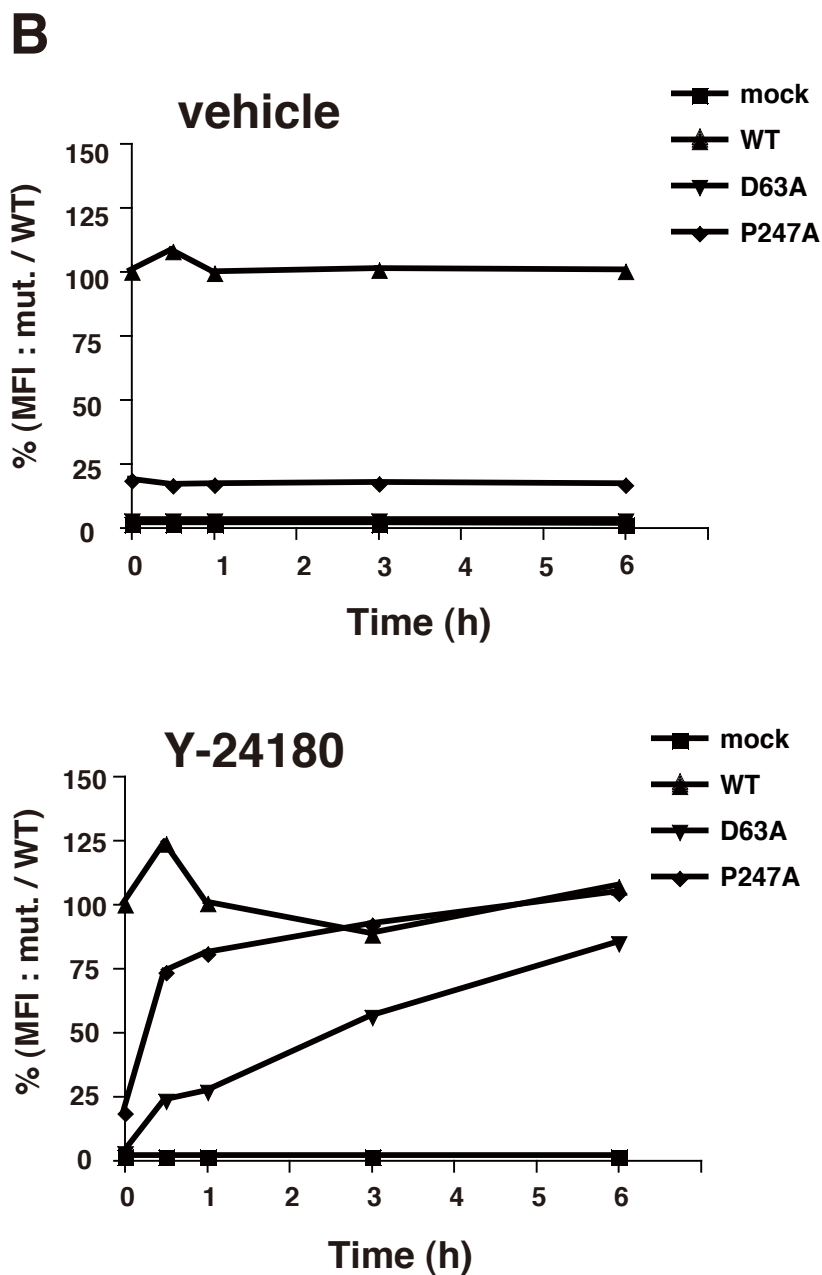
B : Time course of the Y-24180-dependent cell surface expression was examined using HeLa-WT, HeLa-D63A, HeLa-P247A, and HeLa-mock cells.   After treatment of these cells with Dox, Y-24180 (1μM) (*right*) or vehicle (ethanol) (*left*) was added to the culture medium and cell surface expressions of each receptor were measured at the indicated periods.   The expression levels were evaluated by MFI, represented as a percentage of WT HA-hPAFR at 0 time.   The data are representative of three independent experiments with identical results.

Because the ligand-dependent increases in cell surface expression of the mutant receptors were observed within 30 min, these phenomena seemed to be due to the facilitation of ER export of the receptors, rather than the augmentation of protein synthesis.   Thus, to find evidence of the promotion of ER export of the mutant PAFRs by its specific ligands, I used immunofluorescence confocal microscopy to examine the subcellular localization of the mutant PAFRs after treatment with Y-24180.   In these experiments, mutant receptors that had accumulated in the ER disappeared following treatment with Y-24180; they were detected mainly on the plasma membrane (Fig. 10*A*). Next, the enhancement of ER export was elucidated by investigating the glycosylated pattern of the PAFRs.   As shown in Fig. 10*B*, the broad species of the mutant PAFRs, which was not detected in non-treated cells, appeared after treatment with Y-24180, implying that modification of the mutant PAFRs (40-50 kDa) matured by transport to the Golgi apparatus.   In general, misfolded proteins that accumulate in the ER are ubiquitinated and then degraded by proteasomes.   Therefore, I further examined the amount of ubiquitinated receptors with or without Y-24180 treatment using an anti-Ub antibody (41, 42).   Without ligand treatment, the mutant receptors were remarkably ubiquitinated compared with WT HA-hPAFR, whereas ubiquitination was decreased by treatment with Y-24180 (Fig. 11).   Thus, it is possible that the mutations in the conserved residues lead to an aberrant structure of PAFR, resulting in restrained export by the quality control machinery in the ER.   This structural deficiency could be
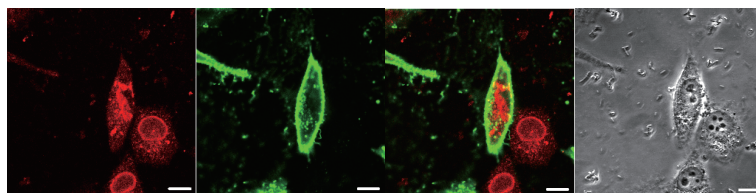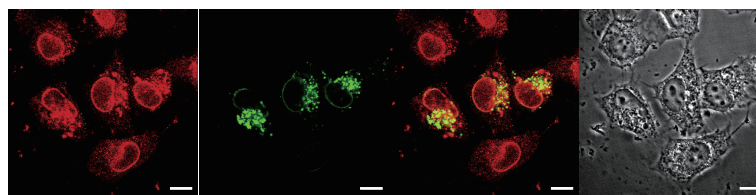
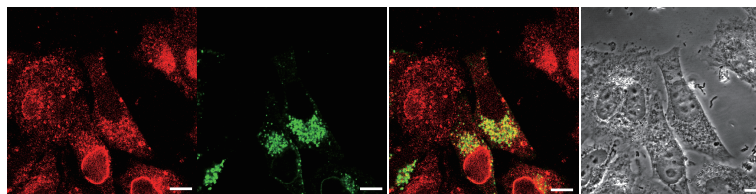**A** vehicle anti-Calreticulin anti-HA merge phase contrast **FIGURE 10.**
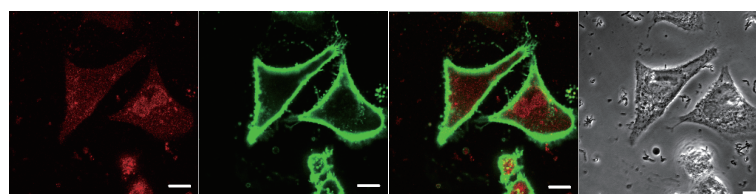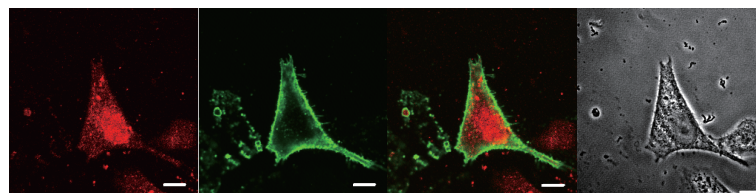
WT

D63A

P247A

Y-24180 anti-Calreticulin anti-HA merge phase contrast
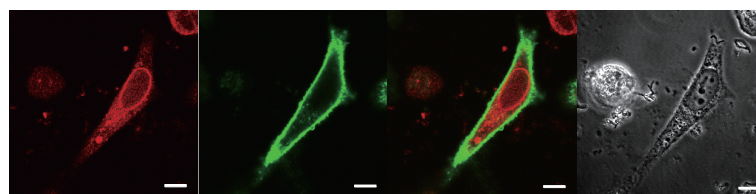
WT

D63A

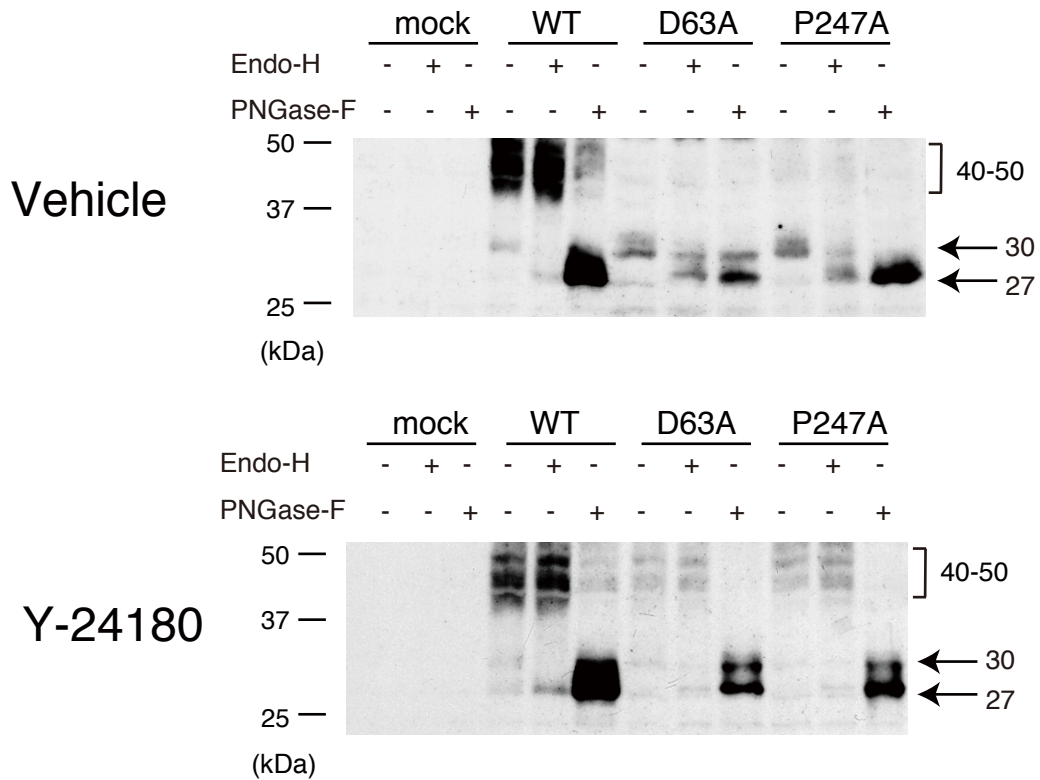P247A

Figure 10.   Export of the m utant HA-hPAFRs from the ER by the pharmacological chaperone.

  A : Effects of Y-24180 (1 µM) on the subcellular localization of WT and mutant HA-hPAFRs were observed by immunofluorescence confocal microscopy. Twenty-four hours after transfection of HeLa cells with WT HA-hPAFR, D63A, or P247A, the cells were treated with vehicle (ethanol) (*upper*) or Y-24180 (*lower*) for 24 h, then subjected to immunocytochemical analysis.   Calreticulin and HA-tagged hPAFRs were visualized using anti-Calreticulin (*red*) and anti-HA (*green*) antibodies, respectively.   White bars in the photos indicate 10 µm.   The data are representative of three independent experiments with identical results.

FIGURE 10.

B

B : Deglycosylation experiments were performed using protein preparations from HeLa-WT, HeLa-D63A, HeLa-P247A, and HeLa-mock cells with (*lower*) or without (*upper*) Y-24180 (1 μM) treatment for 24 h.   Before preparation of the proteins, these cells were treated with Dox.   Deglycosylation assay was per formed as described in Fig. *6B*.   Molecular standards are indicated at the *left* of the gel.   The data are representative of three independent experiments with identical results.
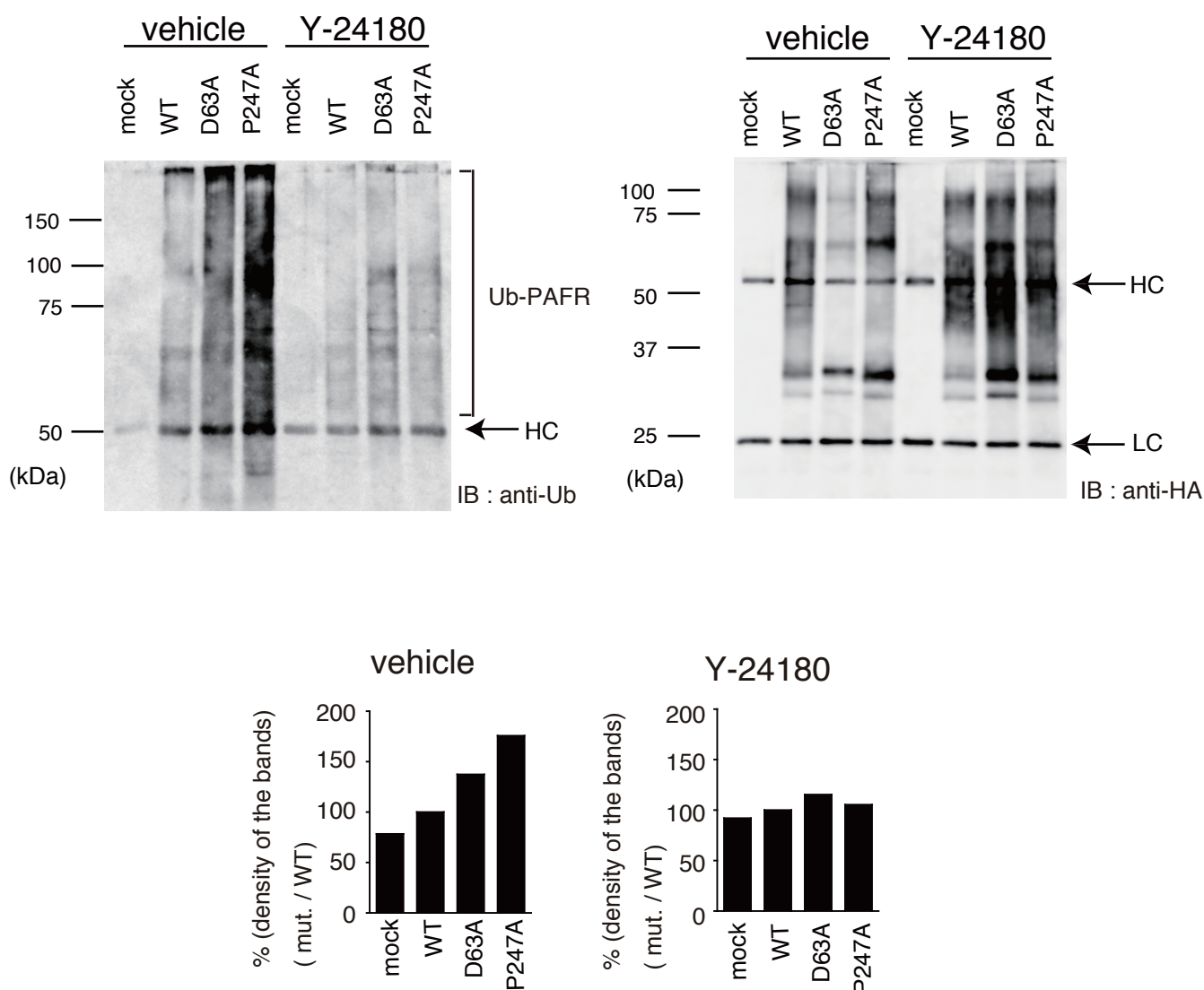
FIGURE 11.

Figure 11.   Decreased ubiquitinated proteins by the effects of pharmacological chaperones.

Effect of Y-24180 on the ubiquitination of WT and mutant HA-hPAFRs was observed using transiently transfected HeLa cells.   Before preparation of the proteins, these cells were incubated with 1 μM of Y-24180 for 24 h.   After immunoprecipitation of the HA-hPAFRs with anti-HA antibody, the precipitated samples were subjected to Western blot analysis using anti-Ub (*left*, 8% polyacrylamide gel) and anti-HA (*right*, 10% polyacrylamide gel) antibodies.   *HC*, heavy chain; *LC*, light chain.   The signal intensities of the ubiquitinated bands shown in the *left panel* were calculated by LAS-4000 mini (FUJIFILM, Tokyo, Japan) and represented as the percentage of that of WT HA-hPAFR (*bottom*).   Molecular standards are indicated at the *left* of the gel.   The data are representative of three independent experiments with identical results.

40

rescued by the binding of ligands, probably due to a stabilization of the conformation and/or enhancement of refolding; therefore, the mutant PAFRs can be exported from the ER.

Functional Analysis of Mutant PAFRs Sorted to the Cell Surface by the Chaperones -

To further gain insight into the pivotal role of the conserved residues, I carried out functional analysis of the mutant PAFRs in living cells after they were sorted to the cell surface following treatment with Y-24180. Followed by washing adequately to remove the ligand, I tested intracellular Ca^{2+} mobilization by stimulation with PAF (32, 33). As shown in Fig. 12*A*, I did not detect any PAF-elicited Ca^{2+} mobilization in D63A-expressing cells, even after cell surface expression of the receptor (Fig. 12*B*). In contrast, an increase in PAF-induced Ca^{2+} mobilization with similar dose-dependency to WT-expressing cells was observed in P247A-expressing cells. These results demonstrate that D63 is quite crucial for the activation of PAFR, and probably because of lacking of coupling with G-proteins. Consequently, deficiency of conserved residues in PAFR inhibits its export from the ER, and treatment with pharmacological chaperones helps it pass the quality control machinery in the ER, even though some of the receptors are dysfunctional.
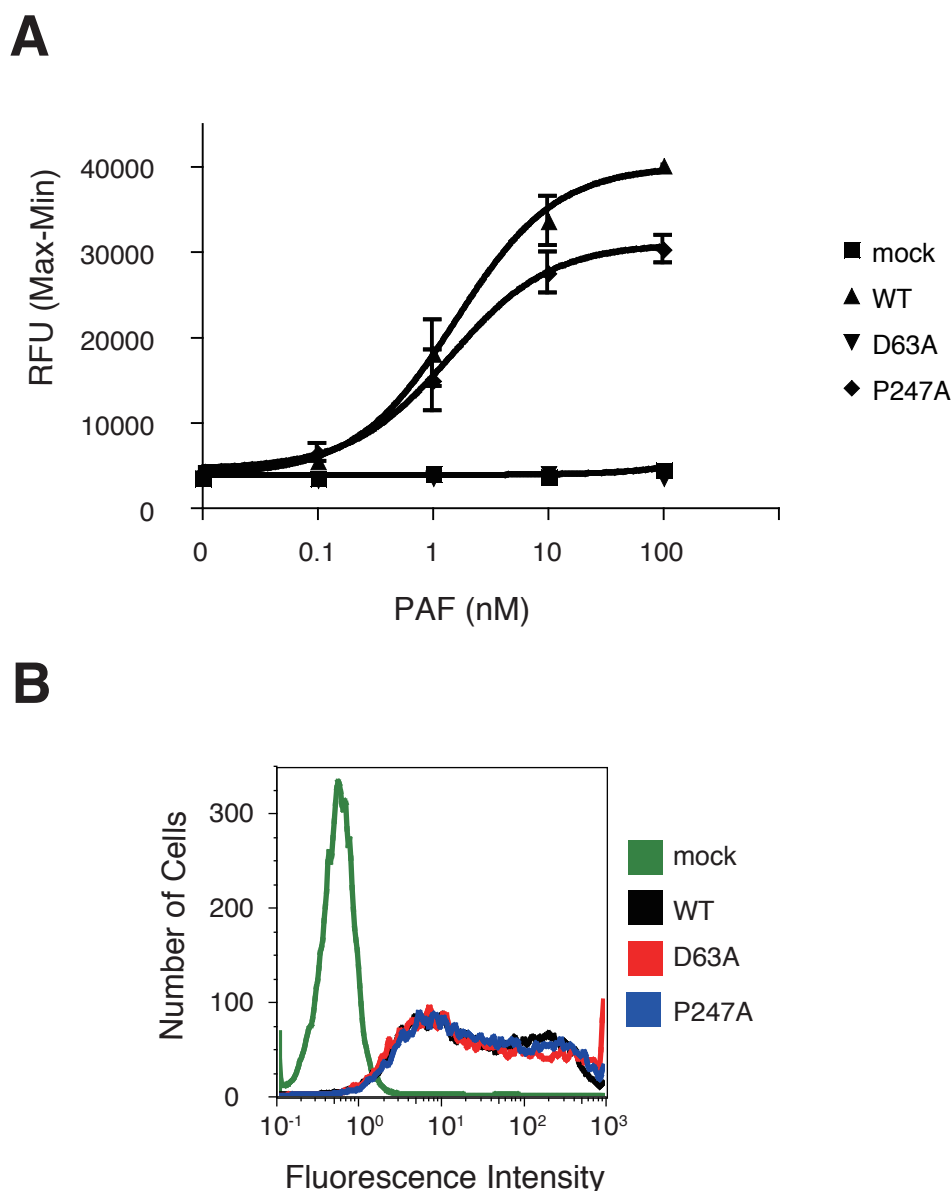
FIGURE 12.

A

B

Figure 12. **Functional analysis of the mutant HA-PAFRs sorted to the cell surface by the pharmacological chaperone.**

A : PAF-elicited intracellular Ca^{2+} increase in HeLa cells transfected with WT HA-hPAFR, D63A, P247 A, or empty vector with Y-24180 (1 μM) treatment was measured by Flex Station.   Relative fluorescence units (RFU, Max – Min) are indicated.   Data are represented as means ± S.D. (n = 3).   The data are representative of three independent experiments with identical results.

B : One of the results from the flow cytometric analysis is shown.   HeLa cells were transiently transfected with WT HA-hPAFR, D63A, P247A, or empty vector, then treated with Y-24180 (1 μM).

Discussion

Although comparison of the amino acid sequences of GPCRs reveals no significant identity among the rhodopsin-type family, many GPCRs of this family share several common residues in the seven transmembrane helices (Fig. 2*A*) (1, 2).   In this report, I generated various mutated PAFRs, in which these residues were substituted by alanines (Fig. 3*A*), to elucidate the importance of the conserved residues in the correct folding of GPCRs during their biosynthesis in the ER.   I successfully identified several residues, including L59, D63, F245, P247, D289, and P290, that are crucial for the trafficking to the cell surface (Fig. 4*A* and *B*).   In particular, mutations of leucine and aspartic acid in TM2 or proline and phenylalanine in TM6 resulted in a drastic decrease in the cell surface expression of the receptors and their accumulation in the ER (Fig. 6*A* and *B*).   In general, helices in a peptide chain tend to be bent at proline residues, and it is likely that mutation of this residue caused a marked conformational change of the receptor.   Indeed, defects in GPCR ligand binding ability due to some mutations of proline residues in TMs have been previously reported (6, 7, 43), suggesting that the proline residues in TMs are quite crucial for the correct folding of GPCRs in the ER.   Likewise, the aspartic acid in TM2 seems to make a hydrogen bond with asparagine in TM1 and alanine in TM7 based on the crystal structure of rhodopsin (12); therefore, it too might contribute to the correct folding of GPCRs.

Interestingly, there have been some reports describing the existence of single nucleotide polymorphism (SNP) mutations of this aspartic acid in TM2 of P2Y5 receptor that lead to diseases such as autosomal ressesive hypotrichosis and autosomal recessive woolly hair (44, 45). Although precise analyses of these mutants have not been completed, these mutant receptors might be eliminated by the ER-associated degradation (ERAD) system because of their aberrant structures. The possibility that mutation of these residues in PAFR resulted in misfolding was further substantiated by examining the capacity of several specific ligands of PAFR to serve as pharmacological chaperones (18-20). In this study, I demonstrated that both ligands, mc-PAF and Y-24180, were able to augment cell surface expression and reduce the ubiquitination of the mutant PAFRs, implying that they enhanced ER export of these mutants (Fig. 8, Fig. 9*A*, *B*, Fig. 10*A, B* and Fig. 11).

Although the binding properties of these ligands to the PAFR mutants had been previously examined using cell-free membrane preparations, which might include the ER membranes (10, 11), little is known about the functional features of these mutants in living cells. To address this issue, I carried out a functional analysis of the mutants after they were trafficked to the cell surface by treatment with Y-24180. In P247A-expressing cells, PAF-elicited Ca^{2+} mobilization was clearly detected (Fig. 12*A*). In contrast, PAF-elicited Ca^{2+} mobilization was not observed in D63A-expressing cells, although a similar amount of the receptors to WT-expressing

cells were trafficked to the cell surface after the Y-24180-treatment (Fig. 12*B*).   Thus,

I propose that the aspartic acid in TM2 is quite pivotal for the activation of coupled

G-proteins on the plasma membrane.   And in the near future we will expect whether

mutant GPCR can couple with G-proteins and make signaling from the structural

analysis of the GPCRs.   Moreover, I provide a useful method for the forced

trafficking of an ER-retained receptor by using its specific ligand.   It could be helpful

in analyzing the function of various misfolded receptors in living cells.

Loss of the GPCR function   ER stress
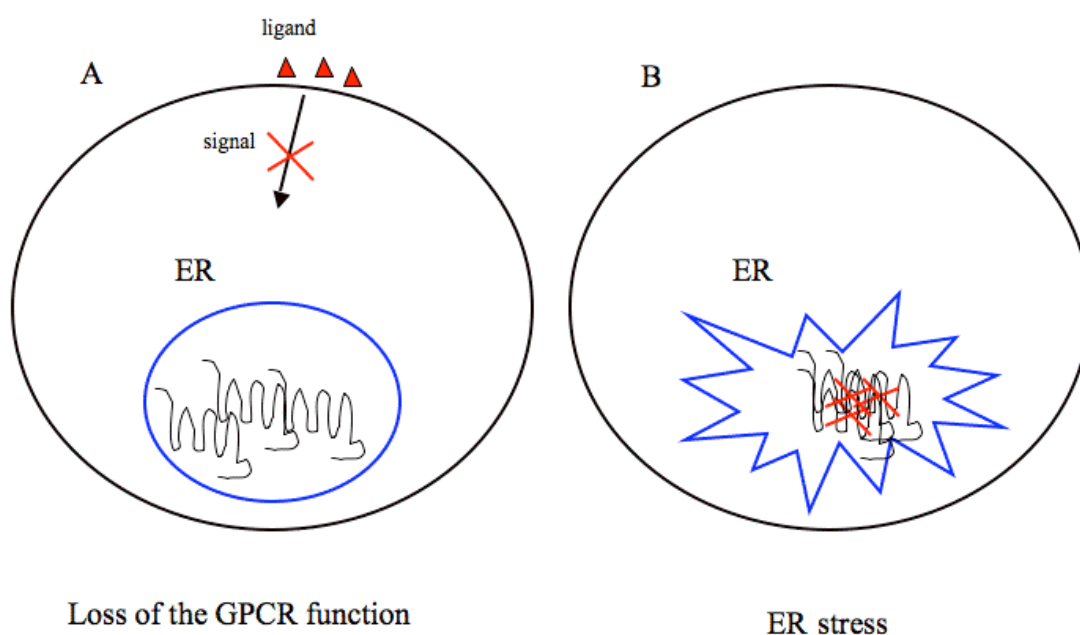
In this work, I observed that most of the mutant PAFRs showed impairment of cell

surface expression, probably due to lack of ER export.   In general, accumulation of

aberrant GPCRs in the ER can lead to two different phenotypes in cells: loss of

function and ER stress.　First, some misfolded GPCRs can be effectively translocated into the cytosol and degraded through ubiquitination by the ERAD system.　Even if those GPCRs still are capable of ligand binding and signal transduction, the trafficking of these receptors to the cell surface is remarkably attenuated, resulting in a loss of function (A).　The consequential deficiencies are similar to the phenotypes observed in genetically deficient mice.　At present, at least 10 congenital diseases have been linked to mutations in GPCRs that lead to their retention in the ER (18-20).　For example, mutations of vasopressin V2 receptor (V2R) and gonadotropin-releasing hormone receptor (GnRHR) cause nephrogenic diabetes insipidus and hypogonadotropic hypogonadism, respectively.　In the case of V2R, more than 175 different mutations distributed throughout the coding lesion of the sequence of this receptor have been identified in patients.　Although some of these mutations involve either losses of ligand binding or G-protein activation, the majority of other "functional mutants" appear to be recognized as misfolded proteins that are retained in the ER by the quality control system before being degraded.　Chemical compounds known as pharmacological chaperones have been identified as stabilizing three of these mutant GPCRs, GnRHR, V2R, and rhodopsin, thus promoting their proper transport to the cell surface (46-48).　Second, continuous accumulation of misfolded proteins, including GPCRs, in the ER can evoke severe ER stress that consequently induces cytotoxicity (49), even if the loss of the GPCRs does not affect cell functions (B).　Here, I present

additional information that GPCRs bearing certain mutations are retained in the ER, and that this ER accumulation could be circumvented by treatment with specific ligands of the receptor. Although little information exists regarding whether SNPs of rhodopsin-type GPCRs lead to ER accumulation and result in severe diseases, including ER stress, such deficiencies might be effectively rescued by treatment with specific ligands, which gain access to the ER lumen by passing through the ER and plasma membranes (50-53). Many diseases may be caused by the accumulation of proteins in the ER because of loss of function or ER stress. So, analysis using pharmacological chaperones may make more drugs to treat these diseases.

Recently, some proteins are reported to work in the trafficking to the cell surface in GPCRs, for example, one of the GPCR-associated sorting proteins, GASP-1 in δ-opioid receptor (54, 55) and Drip78 in dopamine D1 receptor (56). It is possible that some proteins work as chaperones and interactions or affinities with them may have influence in trafficking of PAFR to the cell surface. I could not detect them but the differences of the time to get to the cell surface in each mutant (Fig. 9*B*) may the results from the different affinities.

In this study, I tried to see the interaction of mutant GPCRs and ubiquitin E3 ligase (15-17). I successfully observed that hPAFRs were co-immunoprecipitated with hHRD1 (Fig. 7*B*), which is known to conjugate ubiquitin onto proteins unfolded in transmembrane domains. I performed these experiments by using transiently

transfected cells, so it is of course for Flag-hHRD1 to co- immunoprecipitate even with WT HA-hPAFR, I could not see the difference in the amount of the precipitated Flag-hHRD1 between WT and mutants. So, I gave up this type of assay though I planed to do si-RNA assay of hHRD1.

In these 5 years, BiP was reported to work to keep the homeostasis of the intracellular environment as interactions of other proteins (57). It interacts with misfolded proteins and activates the unfolded protein responses or leads them to be degraded in proteasome. Other than ubiquitin ligases, such proteins may work in degradation of mutants. It is future task to elucidate them.

In summary, my data demonstrate the possibility that the quality control system in the ER recognizes the aberrant structure of rhodopsin-type GPCRs bearing mutations in conserved residues of the TM domains. Some of the mutant GPCRs passed the quality control machinery in the ER by binding pharmacological chaperones. It is still unclear how the ER quality control system discriminates native and mutant receptors. Further studies are needed to address this issue. A structural comparison between native and mutant receptors and identification of recognition factors that can discriminate between the structural differences will be important in elucidating this mechanism.

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