Carlsbad, CA), and human POMT2 cDNA was cloned into pcDNA 3.1/Hygromycin (+) as described previously [1,2]. For each of the mutations (R30A, E44A, R105A /R72A, E86A, R145A) examined in this study, the *POMT1* and *POMT2* gene was modified with a QuickChange Site-Directed Mutagenesis Kit (STRATAGENE, La Jolla, CA) according to the manufacturer's instructions. The mutants were generated with following primer pairs: R30A, 5′-ATGGGGTTA CTGAGCGCGCTGTGGCGACTCACC-3′ and 5′-GGTGAGTCGCCACAGC GCGCTCAGTAACCCCAT-3′; E44A, 5′-CGCGGGCTGTGGTTTTTGACGC AGTATATTATG-3′ and 5′-CATAATATACTGCGTCAAAAACCACAGCCC GCG-3′; R105A 5′-CCTGTGTGGTCCCTGGCCCTGCTGCCAGCACTC-3′ and 5′-GAGTGCTGGCAGCAGGGCCAGGACCACACAGG-3′; R72A, 5′-CTGTCCTTCGCCACCGCCTTCCACCGCTTGGACG-3′ and 5′-CGTCCAA GCGGTGGAAGGCGGTGGCGAAGGACAG-3′; E86A, 5′-CCGCACATC TGTTGGGATGCGACTCACTTTGGAAAAATG-3′ and 5′-CATTTTTCCAA AGTGAGTCGCATCCCAACAGATGTGCGG-3′; R145A, 5′-CAGCTACAT GGGAATGGCAGGATTCTGTGCATTCCTTGGC -3′ and 5′-GCCAAGGA ATGCACAGAATCCTGCCATTCCCATGTAGCTG-3′. All mutant clones were sequenced to confirm the presence of the mutations.

2.2. Cell culture and expression of POMT mutants

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 50 mg/ml streptomycin at 37 °C with 5% CO_2. Expression plasmids were transfected into HEK293T cells using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Cells were incubated for 3 days to produce POMT1 and POMT2 proteins. The cells were homogenized in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM DTT, with protease inhibitor mixture (3 mg/ml pepstatin A, 1 mg/ml leupeptin, 1 mM benzamidine-HCl, 1 mM PMSF). After centrifugation at 900×*g* for 10 min, the supernatant was subjected to ultracentrifugation at 100,000×*g* for 1 h. The precipitate was used as the microsomal fraction. Protein concentration was determined by BCA assay (Thermo Fisher Scientific Inc., Waltham, MA, USA). Microsomal fractions were solubilized with 20 mM Tris–HCl, pH 8.0, 2 mM 2-mercaptoethanol, 10 mM EDTA and 0.5% *n*-octyl-β-D-thioglucoside at 4 °C. After centrifugation at 10,000×*g* for 10 min, the supernatant was used as solubilized supernatant.

2.3. Western blot analysis

The microsomal fractions (20 μg) were separated by SDS–PAGE (7.5% gel) and proteins were transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.05% Tween 20, incubated with anti-POMT2 polyclonal antibody [2] or anti-myc (A-14) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and treated with anti-rabbit IgG conjugated with horseradish peroxidase (HRP) or anti-mouse IgG conjugated with HRP (GE Healthcare Bio-sciences Corp., Piscataway, NJ). Proteins that bound to the antibody were visualized with an ECL kit (GE Healthcare Bio-sciences). As reported previously [1,2], anti-POMT1 and anti-POMT2 polyclonal antibodies did not detect endogenous POMT1 and POMT2, respectively. Each antibody is specific for the respective recombinant protein. That is, they do not cross-react with each other.

2.4. Assay for POMT activity

POMT activity was based on the amount of mannose transferred from Dol-P-Man to a glutathione-S-transferase fusion α-DG (GST-α-DG) as described previously with a slight modification [1]. Briefly, assays were carried out in a 20 μl reaction volume containing 20 mM Tris–HCl (pH 8.0), 100 nM [^{3}H]-labeled Dol-P-Man (Dol-P-[^{3}H]Man, 125,000 dpm/pmol, American Radiolabeled Chemicals, St. Louis, MO), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 10 μg of GST-α-DG, and 80 μg of microsomal membrane fraction. Microsomal fractions were solubilized with buffer containing 20 mM Tris–HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside for 1 h and the reaction was initiated by adding Dol-P-[^{3}H]Man. After 1 h incubation at 25 °C, the reaction was stopped by adding 150 μl of PBS containing 1% Triton X-100, and the reaction mixture was centrifuged at 10,000×*g* for 10 min. The supernatant was removed, mixed with 400 μl of PBS containing 1% Triton X-100 and 10 μl of Glutathione-Sepharose 4B beads (GE Healthcare Bio-sciences), rotated at 4 °C for 1 h, and washed three times with 20 mM Tris–HCl (pH 7.4) containing 0.5% Triton X-100. The radioactivity adsorbed to the beads was measured with a liquid scintillation counter. POMT activities were normalized by protein expression levels.

2.5. Immunoprecipitation

Microsomal fractions were lysed with assay buffer (20 mM Tris–HCl, pH 8.0, 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside) in a final concentration of 2 mg/ml for 5 h at 4 °C. After solubilization, proteins were subjected to centrifugation at 10,000×*g* for 30 min and pre-cleaned with CL-6B Sepharose (Sigma–Aldrich, St. Louis, MO). Pre-cleaned supernatants were mixed with anti-myc (9E10) agarose conjugate (Santa Cruz Biotech) and incubated overnight. After 3 washes with the assay buffer, the agarose beads were suspended in sample buffer. Samples were subjected to Western blot analysis.

3. Results and discussion

In the present study, we attempted to elucidate the importance of three amino acids in the enzymatic activity and complex formation of human POMTs. Previously, Arg^{64}, Glu^{78} and Arg^{138} in ScPmt1p were reported to be critical for enzymatic activity [24]. In particular, Arg^{138} is essential for complex formation with ScPmt2p [24]. The membrane topologies of human POMT1 (Fig. 1A) and POMT2 (Fig. 1B) were predicted from bioinformatics analyses [25]. The corresponding three amino acids are conserved in human POMT1 and POMT2 (Fig. 1C). Therefore, we substituted Arg^{30}, Glu^{44} and Arg^{105} in human POMT1 and Arg^{72}, Glu^{86} and Arg^{145} in human POMT2 with Ala by site-direct mutagenesis and expressed the genes in HEK293T cells with their wild-type partners.

Western blots showed that both the POMT1 mutants (Fig. 2A) and the POMT2 mutants (Fig. 3A) were expressed normally, and their mobilities in SDS–PAGE were not affected by the amino acid substitutions. All mutated POMT1s and POMT2s were co-precipitated with wild-type POMT2 or wild-type POMT1, respectively (Figs. 2B and 3B). Each protein expression level of transfectants and the amount of proteins in the immunoprecipitates were determined densitometrically. Low precipitation of R145A-mutant (Fig. 3B) seems to be due to low expression level of protein (Fig. 3A). The expression ratio of R145A-POMT2/POMT1 was about 60% of wild-type POMT2/POMT1. The precipitated ratio of R145A-POMT2/POMT1 was about 70% of wild-type POMT2/POMT1. The expressed POMT2/POMT1 ratios of other mutants were around 80% to 120% and precipitated POMT2/POMT1 ratios were comparable to those of wild-type POMT2/POMT1. Taken together, three amino acids substituted in human POMT1 and POMT2 are not essential for POMT1–POMT2 complex formation. In ScPmt1p, Arg^{138} is essential for complex formation with ScPmt2p [24]. Therefore, changing this residue to Ala caused loss

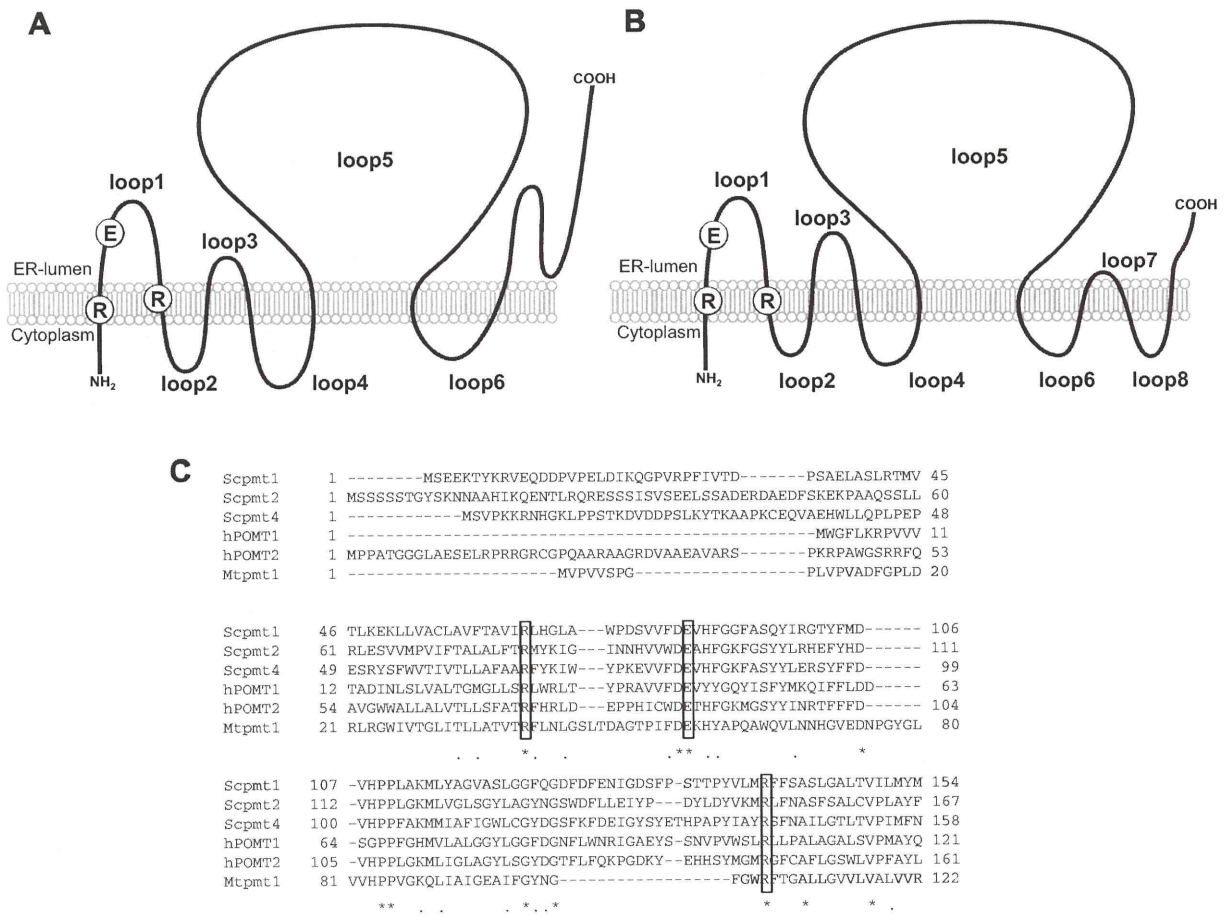

Fig. 1. Schematic representation of human POMT1 and POMT2, and alignment of PMT family members. (A) and (B), Topology models of human POMT1 and POMT2 according to [25]. R, E and R indicate the sites where Arg, Glu and Arg were substituted with Ala by site-directed mutagenesis. The major splicing variant of human POMT1, which lacks amino acids 234–255, was used in this study [2]. (C), ClustalW alignment of *N*-terminal amino acid sequences of protein *O*-mannosyltransferases. ScPmt, *S. cerevisiae* Pmt; hPOMT, human POMT; Mtpmt, *M. tuberculosis* pmt. Dots indicate similar amino acids. Asterisks indicate conserved amino acids. Squares indicate amino acids substituted to Ala.

of enzymatic activity. However, in human POMT1, the same amino acid substitution did not affect POMT1–POMT2 complex formation. These results suggest that Arg^{138} of ScPmt1p and Arg^{105} of human POMT1 have different roles. A phylogenetic analysis showed that the PMT family has three subfamilies: PMT1, PMT2, and PMT4 [12]. ScPmt1p and ScPmt2p are members of the PMT1 and PMT2 subfamilies, respectively. On the other hand, human POMT1 and POMT2 are members of the PMT4 and PMT2 subfamilies, respectively. The members of the PMT1 subfamily form complexes with members of the PMT2 subfamily, and a single member of the PMT4 subfamily (ScPmt4p) forms a homomeric complex and does not form a complex with ScPmt2p [21]. Although *S. cerevisiae* has at least six PMT family members, humans have only two PMT members, POMT1 and POMT2. Therefore, POMT1 and POMT2 must form a heterodimeric complex to have mannosyl transfer activity. This may explain why Arg^{138} of ScPmt1p and Arg^{105} of human POMT1 have different roles.

Next, we examined the effect of amino acid substitution on protein *O*-mannosyltransferase activity. Each of the substitutions in human POMT1 abolished most of the activity (Fig. 2C), indicating that Arg^{30}, Glu^{44} and Arg^{105} in loop1 of POMT1 are essential for POMT activity. On the other hand, the POMT2 mutants retained enzymatic activities (Fig. 3C). R72A-mutant and E86A-mutant sustained about 65% and 75% activity of wild-type POMT, respectively, and R145A-mutant retained enzymatic activity entirely. These results clearly show that these three amino acids play different roles in POMT1 and POMT2.

It is not clear why human protein *O*-mannosyltransferase activity requires co-expression of two components, POMT1 and POMT2 [1,2]. One possibility is that assembly of POMT1 and POMT2 creates a catalytic domain but each POMT1 or POMT2 itself does not have any catalytic activity. Another possibility is that POMT1 or POMT2 is a catalytic molecule and another component may enhance its activity. Recently, we found that zebra fish POMT2 alone expressed in HEK293T cells showed weak activity, while zebra fish POMT1 alone showed no activity [26], suggesting that zebra fish POMT2 itself has enzymatic activity, or may form a complex with endogenous POMT1 resulting in low levels of enzymatic activity. In addition, dermal fibroblast cells from WWS patients with mutations in *POMT1* showed low residual activity [10,12], suggesting that POMT2 has low levels of activity without POMT1. Taken together, these findings indicate that human POMT1 and POMT2 are required for efficient mannose transfer, although they are probably not functionally equivalent.

It is not clear whether loops 1 of ScPmt2p and ScPmt1p have similar roles. Although the substitutions in loop 1 of human POMT2 had little effect on transferase activity (Fig. 3C), POMT activity of lymphoblasts of a WWS patient homozygous for a Tyr96Cys mutation in loop 1 of POMT2 was extremely low [9]. This

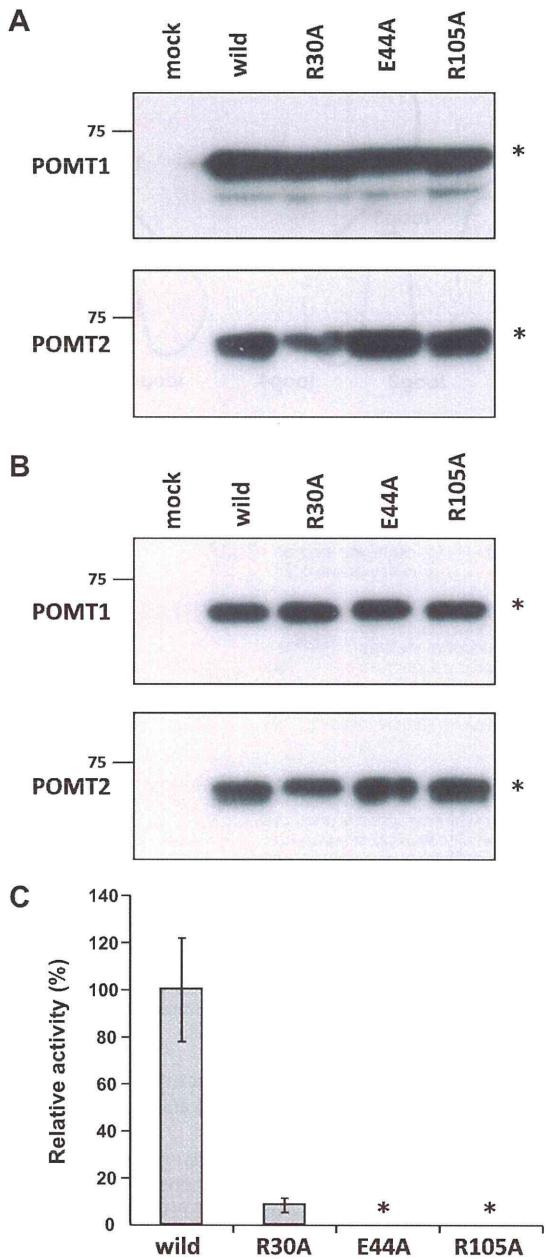

Fig. 2. Effects of amino acid substitutions in POMT1 on POMT activity and complex formation. (A), Western blot analyses of the microsomal fractions from cells co-transfected with POMT1-myc mutants and POMT2. (B), POMT1–POMT2 complex formation *in vivo*. POMT1-myc mutants and wild-type POMT2 were transfected into HEK293T cells and immunoprecipitated by anti-myc (9E10) antibody-conjugated agarose. The resulting precipitates were analyzed by immunoblotting with anti-myc antibody (A-14) and anti-POMT2 antibody. (C), POMT activities of the POMT1-myc mutants co-expressed with POMT2. wild (wild-type POMT1-myc and wild-type POMT2); R30A (R30A-mutant POMT1-myc and POMT2); E44A (E44A-mutant POMT1-myc and POMT2); R105A (R105A-mutant POMT1-myc and POMT2). Asterisks in (A) and (B) indicate the migration positions of each POMT1-myc protein and POMT2 protein. Molecular weight standards are shown on the left. POMT activity was based on the amount of mannose transferred to a GST-αDG. Average values of three independent experiments are shown. POMT activities were normalized by protein expression levels. Asterisks in (C), No activities were detected.

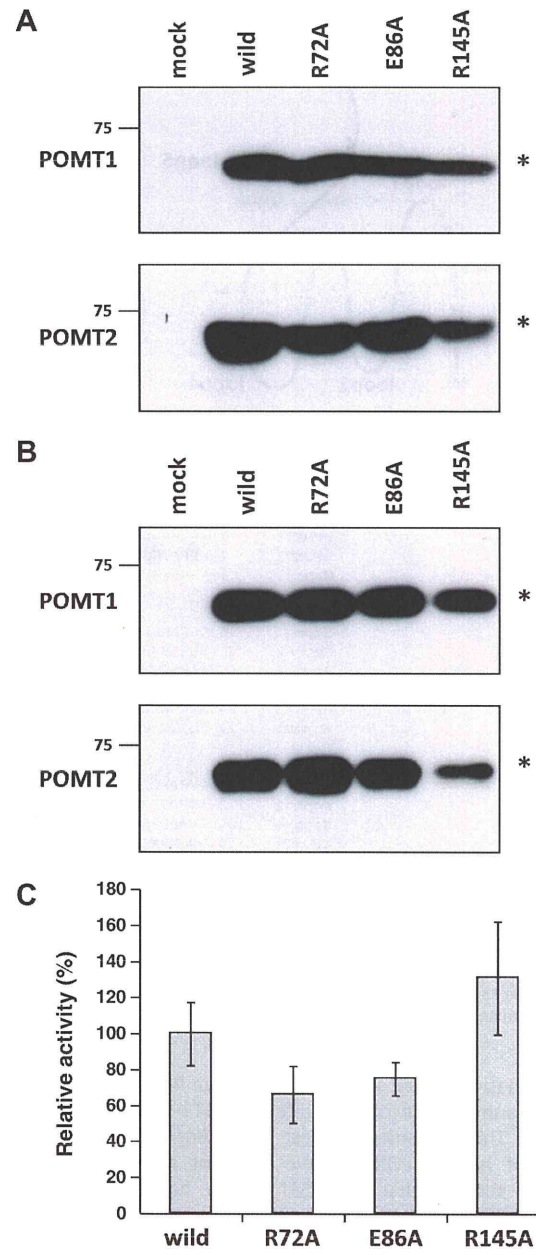

Fig. 3. Effects of amino acid substitutions in POMT2 on POMT activity and complex formation. (A) Western blot analyses of the microsomal fractions from the cells co-transfected with POMT1-myc and POMT2 mutants. (B) POMT1–POMT2 complex formation *in vivo*. POMT1-myc and POMT2 mutants were transfected into HEK293T cells and immunoprecipitated by anti-myc (9E10) antibody-conjugated agarose. The resulting precipitates were analyzed by immunoblotting with anti-myc antibody (A-14) and anti-POMT2 antibody. (C) POMT activities of the POMT1-myc co-expressed with POMT2 mutants. wild (wild-type POMT1-myc and wild-type POMT2); R72A (POMT1-myc and R72A-mutant POMT2); E86A (POMT1-myc and E86A-mutant POMT2); R145A (POMT1-myc and R145A-mutant POMT2). Asterisks indicate the migration positions of each POMT1-myc protein and POMT2 protein. Molecular weight standards are shown on the left. POMT activity was based on the amount of mannose transferred to a GST-αDG. Average values of three independent experiments are shown. POMT activities were normalized by protein expression levels.

suggests that loop 1 of human POMT2 is required for enzymatic activity but its crucial amino acid(s) may be different from those in POMT1.

Mycobacterium tuberculosis has a PMT homolog (Mtpmt) in which the three amino acids corresponding to Arg^{64}, Glu^{78} and Arg^{138} of ScPmt1p (boxed in Fig. 1C) are conserved [11]. Mtpmt catalyzed the initiation of protein mannosylation and Glu^{56} residue

(corresponding to Glu^{78} in ScPmt1p, Fig. 1C) was necessary for enzymatic activity [11]. Since there is not another PMT homolog in *M. tuberculosis*, Mtpmt may function as a homomeric complex or form a complex with other components to show enzymatic activity. The sequence similarity and the fact that a Glu residue in loop 1 is required for enzymatic activity suggest that protein *O*-mannosylation is conserved from bacteria to eukaryotes including human.

In the present study, we found that some of the amino acids that are critical for activity and complex formation are different in human POMT1 and POMT2. To better understand the mechanism of protein *O*-mannosylation, it will be necessary to study the structure of the POMT1–POMT2 complex. Such studies will help to determine the spatial relation among these critical amino acids.

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