N-glycosylation is required for efficient secretion of a novel human secreted glycoprotein, hPAP21

Yu-Bo Zhou[a,b], Feng Liu[b], Zhi-Dong Zhu[b], Hong Zhu[b], Xin Zhang[b], Zhi-Qin Wang[b], Jian-Hua Liu[a], Ze-Guang Han[b,*]

[a] *College of Life Science and Biotechnology, Shanghai Jiaotong University, 1954 Huashan Road, Shanghai 200030, China*
[b] *Chinese National Human Genome Center at Shanghai, 351 Guo Shou-Jing Road, Shanghai 201203, China*

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Abstract The present study reported the isolation and characterization of a novel human secreted protein, named as hPAP21 (human protease-associated domain-containing protein, 21 kDa), encoded by the hypothetical gene chromosome 2 open reading frame 7 (*C2orf7*) that contains signal peptide in its N-terminus, without transmembrane domain, except for PA domain in its middle. Western blotting assay indicated that the c-Myc tagged hPAP21 could be secreted into culture medium in the transfected Chinese hamster ovary cells. However, the molecular weights, whatever intracellular (28 kDa) or extracellular (30 kDa) forms, are larger than that of the prediction. To define whether the glycosylation was important process for its secretion, endoglycosidase H (Endo H) and PNGase F (PNG F) were employed to evaluate the effect of glycosylation types on secretion of hPAP21. Interestingly, the extracellular forms were primarily sensitive to PNG F, not Endo H, implying that complex N-glycosylation could be required for the secretion of hPAP21. Furthermore, N-glycosylation of Asn171 was confirmed as potential crucial process for the secretory protein via site-directed mutagenesis assay. All data will be contributed to the understanding of molecular functions of hPAP21.

Keywords: Human protease-associated domain-containing protein, 21 kDa; Chromosome 2 open reading frame 7; Glycosylation; Secretion

1. Introduction

About 10% of the whole human proteins, including cytokines, hormones, digestive enzymes, and immunoglobulins, are secretory proteins [1]. These proteins play important roles in many fundamental cellular and organism functions, such as immune defense, intercellular communication, morphogenesis, angiogenesis, apoptosis, and cell differentiation [2]. Moreover, human tissue-specific plasminogen activators, erythropoietins, peptide hormones and digestive enzymes account for the large majority of protein therapeutics [3].

Post-translational modifications are common in eukaryotic secreted proteins. The most common and complex post-translational modification is glycosylation that could be relevant to about 1% of genes in the mammalian genome although there are 20 post-translational modifications in eukaryotic cells [4–6]. Glycosylation appears to be very conserved from yeast to humans, and more, over half of the glycosylated proteins are reported to have N-linked oligosaccharides. The N-linked glycosylations could facilitate protein folding, protect proteins from proteolysis, and guide protein trafficking; meanwhile, the glycans of secreted proteins are important for secretion [7–12].

Apparently, identification for the genes encoding secreted proteins will be very significant, not only for understanding their roles in biological processes, but also for the potential applications in therapeutics. Recently, we performed a strategy to predict potentially secreted proteins based on bioinformatics tools and human genomic information, and then the predicted genes were isolated and characterized through further experiments [13]. In this study, a novel human protease-associated domain-containing protein, 21 kDa (hPAP21), encoded by the hypothetical gene chromosome 2 open reading frame 7 (*C2orf7*), was also screened out as a candidate that was confirmed to be secreted into culture medium (CM) in vitro, and N-glycosylation could be important to its secretion.

2. Materials and methods

2.1. Bioinformatics analysis

Bioinformatic prediction was performed as described previously [13] by using softwares, including SignalP v.2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0/), PSORT II (http://psort.nibb.ac.jp/) and SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), which were used for predicting signal peptide, subcellular localization, hydrophobicity and transmembrane region, respectively. The homologous analyses of the candidate secretory proteins were carried out via BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) programs, while modification sites of proteins were predicted through PROSITE (http://us.expasy.org/prosite/). Multiple sequence alignment and phylogenic analysis were performed using ClustalW and MEGA2 programs.

2.2. RNA extraction and RT-PCR

Total RNA was extracted with TRIzol reagent (Life Technologies Inc., Gaithersburg, MD) according to the protocol recommended by the manufacturer. Reverse transcription was carried out at 42 °C for

* Corresponding author. Fax: +86-21-50800402.
E-mail address: hanzg@chgc.sh.cn (Z.-G. Han).

Abbreviations: C2orf7, chromosome 2 open reading frame 7; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; Endo H, endoglycosidase H; PNG F, PNGase F; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; aa, amino acid; CL, cell lysate; CM, culture medium

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1 h in a 25 μl reaction mixture that contained 2.5 μg of total RNA, 10 pmol of oligo(dT), and 200 unit of M-MLV Reverse Transcriptase (Promega, Madison, USA). The entire open reading frame (ORF) of *C2orf7* was amplified by polymerase chain reaction (PCR) with the designed primers. PCR mixture was preheated at 94 °C for 4 min, and then thermal-cycled for 35 cycles (94 °C for 40 s, 60 °C for 30 s, and 72 °C for 50 s), and ended with a final extension at 72 °C for 7 min.

2.3. Expression vector construction

Wild-type hPAP21 with entire ORF was inserted into mammalian expression vector pcDNA3.1-Myc-HisA (−) vector (Invitrogen, San Diego, CA) through amplifying DNA fragment using PCR, in order to generate the hPAP21-Myc fusion protein with c-Myc tag at the C-terminus. Site-directed mutagenesis was used to generate the mutated proteins (Fig. 1A) based on PCR method [14]. In brief, the consensus sequence of glycosylation, Asn-Xaa-Ser/Thr, was mutated by creating codons for Gln (CAA) instead of Asn (AAT). Wild-type and mutants' primers were the following (underline indicated the mutated sites): forward primer for wild-type (W-F-P): 5′-CCGGATCCATGGTCCCCGGCGC-3′; reverse primer for wild-type (W-R-P): 5′-CCAAGCTTCCAGAAGGTCCAGGGC-3′; forward primer for mutant 1(Asn121Gln) (M-1-F-P): 5′-GACAACGCAGTTGAC**CAA**GACAGCTTCTACG-3′; reverse primer for mutant 1 (M-1-R-P): 5′-CGTAGAAGCTGTC**TTG**GTCAACTGCGTTGTC-3′; reverse primer for mutant 2(Asn171Gln)(M-2-R-P): 5′-CCAAGCTTCCAGAAGGTCCAGGGCGGTTGCAGCAGCTCAAAGGTGGGGATGCTGGTGAC**TTG**GACTGGGATGGAAATGATGGCCC-3′. Two DNA fragments were amplified, one by the paired primers M-1-F-P and W-R-P, as well as the other by W-F-P and M-1-R-P, which wild-type hPAP21 was used as template. The PCR products were purified with MiniElute™ Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), respectively, and then mixed as template for the second PCR through the primers W-F-P and W-R-P. The purified PCR product was inserted into pcDNA3.1-Myc-HisA (−) vector to generate a recombinant plasmid pcDNA3.1A-hPAP21-mutant 1-Myc. Similarly, recombinant plasmid pcDNA3.1A-hPAP21-mutant 2-Myc also was generated using W-F-P and M-2-R-P primers, which wild-type hPAP21 was used as template. The recombinant plasmid containing mutant 3 (Asn121Gln and Asn171Gln) with both mutated sites was constructed by PCR with primers W-F-P and M-2-R-P, which vector pcDNA3.1A-hPAP21-mutant 1-Myc was used as template. The PCR condition was done as above and products were analyzed by electrophoresis on a 2% agarose gel. All constructs were confirmed by DNA sequencing.

2.4. Northern blot analysis

Human Multiple Tissue Northern Blot (Clontech, Polo Alto, CA) membrane was hybridized according to the manufacturer's protocol, which partial *hPAP21* (60–564 nt) PCR product was used as probe that was ^{32}P-labeled with Random Primer DNA Labeling Kit (TaKaRa Inc, DaLian, China). After hybridization, the membrane was washed in 2× sodium saline citrate (SSC), 0.05% sodium dodecyl sulfate (SDS) at room temperature for 3× (30 min each time), and then in 0.1× sodium saline citrate (SSC), 0.1% SDS for 2 times (30 minutes each time) at 50 °C, and finally visualized by autoradiography.

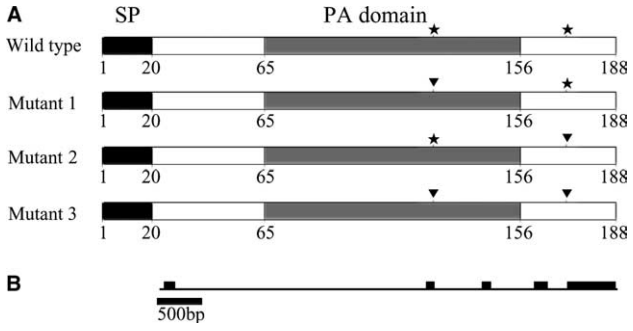

Fig. 1. The representative structure of the hPAP21 (C2orf7). (A) Schematic structure of wild-type hPAP21 and its mutants. SP, signal peptide; PA domain, protease-associated domain. Asterisks indicate the potential N-glycosylation sites; triangles indicate Asn residues mutated to Gln. The numbers below the schematic structure indicate the length of the protein. (B) Schematic genomic structure of the *hPAP21* (*C2orf7*). Black boxes represent exons, while horizontal lines represent introns.

2.5. Cell transfection and selection of stably transfected cells

The recombinant plasmids containing wild-type hPAP21 or mutants were transiently transfected into Chinese hamster ovary (CHO) cells (1.5×10^5 cells/35 mm dish) with LipofectAMINE reagents (Gibco-BRL), respectively, according to the manufacturer's protocol. The CHO cells were cultured and grown overnight in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum (FBS) by 5% CO_2 at 37 °C. For selection of stably transfected subclones, G418 (1.0 mg/ml) (Life Technologies) was supplemented for 3 days after cell transfection, and then the cells were continuously cultured in 10% FBS/DMEM for 3 weeks. The subclones were selected, expanded and detected by Western blotting with antibody 9E10 (Clontech) against c-Myc tag.

2.6. Western blot analysis

After the CM of CHO cells transfected with recombinant plasmids was collected, the remaining cells were washed with cold phosphate-buffered saline (PBS) (pH 7.4) two times (5 min each time) and lysed with lysis buffer (50 mmol/L Tris–HCl, pH 8.0, 150 mmol/L NaCl, 100 μg/ml PMSF, and 1% Triton-X 100) on ice for 30 min. The CM and the cell lysates (CL) were purified using TALON metal affinity resins (Clontech) according to the manufacturer's manual. Purified proteins were separated by 15% SDS–polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto a PVDF membrane (Amersham Pharmacia Biotech AB, Uppsala, Sweden). After being blocked by PBS containing 2% bovine serum albumin (BSA), 3% non-fat milk and 0.1% Tween 20 overnight at 4 °C, the membrane was incubated with monoclonal antibody against human c-Myc (9E10) (Clontech) (1:1000 dilution) at room temperature for 2 h, and followed by incubation with a horseradish peroxidase-linked goat anti-mouse secondary antibody (Gibco-BRL) (1:10 000 dilution) at room temperature for 2 h. The signals were detected with the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech).

2.7. Subcellular localization

Cells cultured on polylysine-treated slides were co-transfected with 2 μg of plasmids pcDNA3.1-hPAP21-Myc and pEYFP-Golgi (Clontech) using LipofectAMINE (Gibco BRL). After 60 h, the transfected cells were washed 2× PBS and fixed with 4% paraformaldehyde/0.1%Triton-X 100 in PBS on ice for 30 min. The cells were immunostained with monoclonal antibody to c-Myc (9E10) (Clontech) (1:200 dilution) at room temperature for 2 h after being blocked in PBS containing 5% BSA overnight at 4 °C, and followed by incubation with goat anti-mouse antibody–Cy5 conjugate (Biological Detection Systems, Inc, Pittsburgh, PA, USA) (1:400 dilution) at room temperature for 2 h. The slides were viewed by confocal scanning microscopy (Leica Microsystems Heidelberg GmbH, Germany).

2.8. Deglycosylation experiments

The purified proteins from CL or CM were pre-denatured in 1× Glycoprotein Denaturing Buffer at 100 °C for 10 minutes. According to the manufacturer's instructions, the denatured proteins were treated with PNGase F (PNG F) (New England Biolabs Inc, Beverly, MA, USA) or Endo H (New England Biolabs), respectively, at 37 °C overnight. The digested proteins were analyzed by Western blotting assay after separated on 15% SDS–PAGE.

2.9. Tunicamycin treatment

The stable transfected cell line with exogenous hPAP21 and its variants was seeded at 5×10^5/35-mm dish and cultured for 24 h, and then the medium was replaced with fresh medium containing tunicamycin (Sigma, St. Louis, MO) (2.0 μg/ml) or the same volume of dimethyl sulfoxide and cultured for additional 24 h. The proteins purified from CL and CM were analyzed with Western blotting assay.

3. Results

3.1. Isolation and characterization of hPAP21

hPAP21, encoded by the hypothetical gene *C2orf7* (GenBank Accession No. NM 032319), was screened out as potential secretory protein using the previous strategy [13]. Analyses of SignalP and SOSUI softwares implied that the hPAP21 protein has a significant signal peptide in its N-terminus (aa: 1–20) (Fig. 1A), without transmembrane regions. Furthermore, the possibility of extracellular localization is 66.7%, much higher

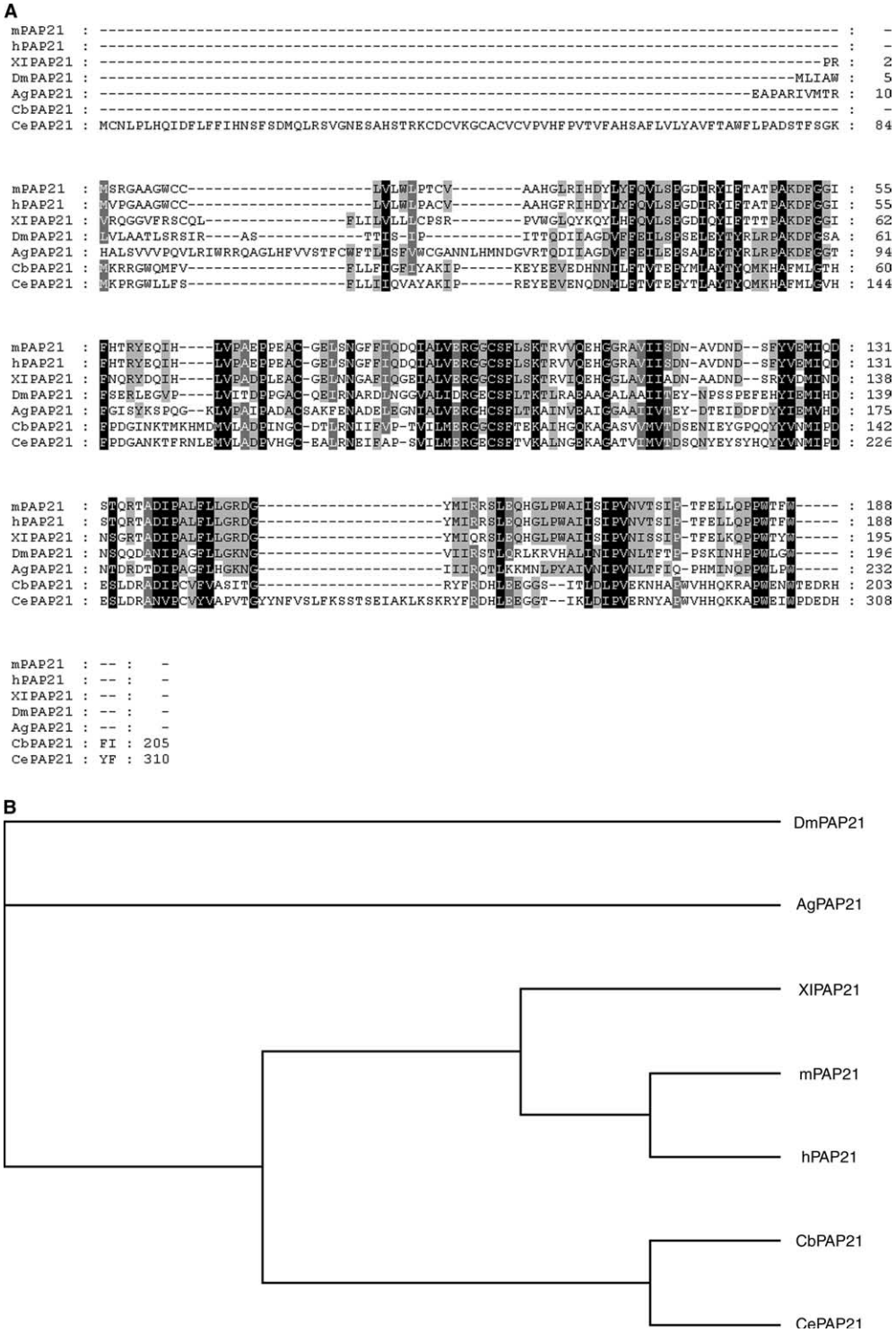

Fig. 2. Phylogenetic relationship between hPAP21 and some associated proteins. (A) Multiple aa sequence alignment of hPAP21 and some homologs. Identical residues are in black and conserved substitutions are in gray. The numbers of right side represent the length of each sequence. (B) Phylogenetic tree of hPAP21 and its orthologs. mPAP21, mouse PAP21 (XP_132652); DmPAP21, *Drosophila melanogaster* PAP21 (NP_611740); AgPAP21, *Anopheles gambiae* PAP21 (XP_314522); XlPAP21, *Xenopus laevis* PAP21 (AAH70658); CbPAP21, *Caenorhabditis briggsae* PAP21 (CAE68661); CePAP21, *C. elegans* PAP21 (NP_l508831).

than those of vacuolar (22.2%) and mitochondrial (11.1%), which hPAP21 were predicted by the PSORT II program. All the computational predictions suggested that the hypothetical gene *hPAP21* (*C2orf7*) might encode a secretory protein albeit the hypothesis needs to be confirmed.

hPAP21 (*C2orf7*) has five exons and four introns, spanning 5244 bp in human genome context (Fig. 1B) although *hPAP21* only encodes 188 amino acid (aa) residues with a PA domain (Fig. 1A). Interestingly, hPAP21 might have its orthologs from worm to mouse, sharing 41–97% identity of aa residues (Fig. 2A). The phylogenic analysis suggested that *PAP21* could have common ancestor and, moreover, human and mouse PAP21 seem to be more closer evolutionally to that of African clawed frog and worms, rather than insects such as fruit fly and mosquito (Fig. 2B).

Furthermore, to evaluate tissue expression pattern of *hPAP21* (*C2orf7*), Northern blot analysis was performed using Human Multiple Tissue Northern Blot membrane (Clontech). The data indicated that the gene was highly expressed in skeletal muscle, heart and liver, as well as moderate in kidney, only with single transcript of about 1.1 kb (Fig. 3).

3.2. Expression and secretion of hPAP21

To define whether hPAP21 could be secreted into extracellular medium, recombinant plasmid pcDNA3.1-hPAP21-Myc was transiently and stably transfected into CHO cells. Interestingly, the c-Myc-His tagged hPAP21 fusion protein was detected by antibody against c-Myc in both CM and CL via Western blotting assay after affinity purification by Ni^{2+} resin (Fig. 4), demonstrating that *hPAP21 (C2orf7)* indeed encodes a secretory protein. However, two main bands, approximately 30 kDa, of hPAP21 fusion protein in CM, as shown in Fig. 4 (indicated by 'a' and 'b' arrows), were significantly larger than those in CL (Fig. 4, indicated by 'c' arrow), implying that post-translational modification could be pivotal process during the secretion of hPAP21.

3.3. N-glycosylation state of hPAP21

PROSITE program prediction suggested that the aa residues 121–124 (NDSF) and 171–174 (NVTS) of hPAP21 could have two potential N-glycosylation modification sites. To define the

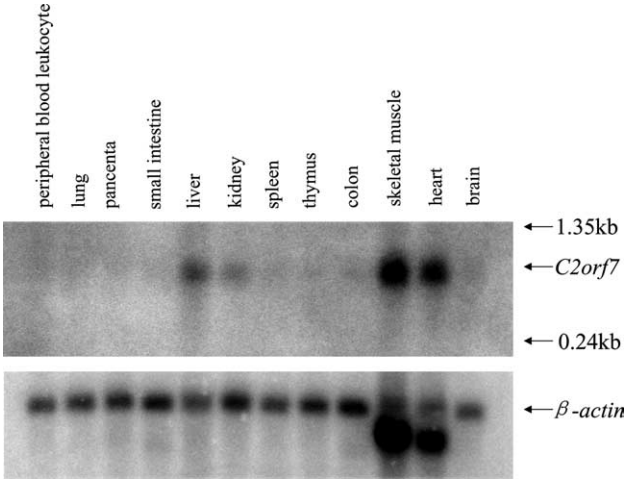

Fig. 3. Tissue expression pattern of *hPAP21* (*C2orf7*). Human Multiple Tissue Northern Blot membranes (Clontech) were hybridized with *hPAP21* (*C2orf7*) cDNA probe.β-Actin was used as control.

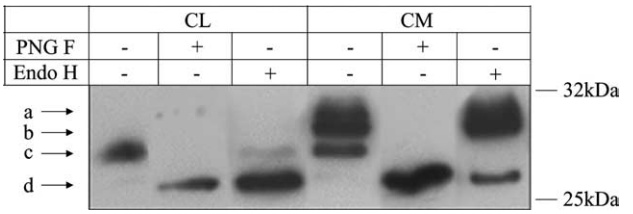

Fig. 4. The analyses of glycosylation type of intracellular and extracellular hPAP21. Protein was harvested from CL and CM that derived from stably transfected CHO cells with exogenous hPAP21, and then digested with PNG F and Endo H, respectively, after purification by TALON metal affinity resins. "a–c" arrows indicate the different bands with distinct sizes from CL and CM with (+) or without (−) enzymes treatment (PNG F; Endo H).

issue, PNG F and Endo H, which can cleave N-linked oligosaccharide structures from Asn intact, as well as chitobiose core of high mannose and some hybrid oligosaccharides from N-

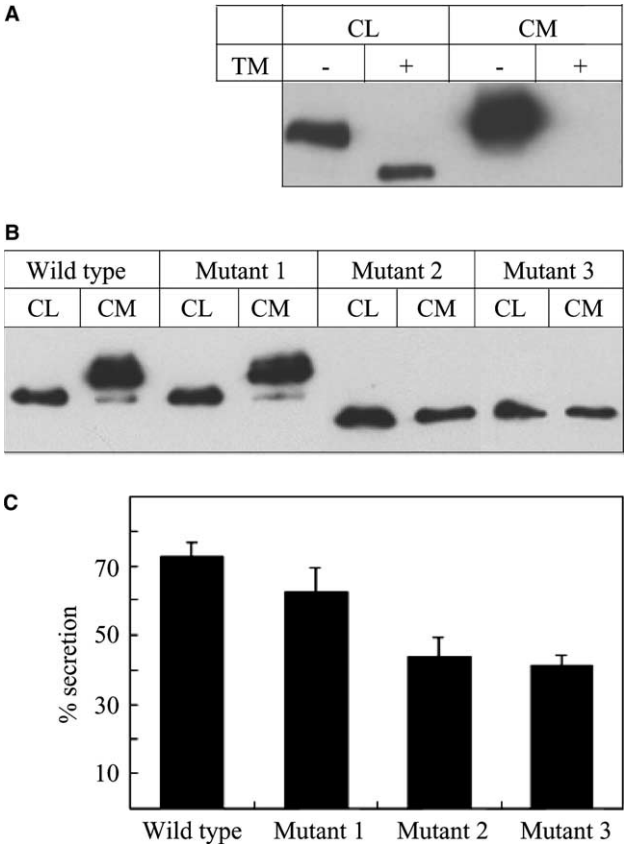

Fig. 5. The effect of N-glycosylation on the secretion of hPAP21. (A) The effect of tunicamycin, an inhibitor of N-glycosylation, on biosynthesis and secretion of wild-type hPAP21. The CHO cells transfected with wild-type hPAP21 were treated with (+) or without (−) tunicamycin for 24 h, and then protein derived from CL and CM was analyzed, respectively, through Western blotting assay. TM, tunicamycin. (B) The effect of potential glycosylation sites on the secretion of hPAP21. The mutants were constructed via site-directed mutagenesis assay and transfected into CHO cells, whereas the plasmid containing wild type hPAP21 was used as control. (C) The evaluation of secreted efficiency between wild-type and mutants of hPAP21. The percentage of secreted protein was determined by density ratio of secreted forms to both intracellular and extracellular forms through densitometry scans on Western blotting. The experiments were repeated three times. Data values and error bars showed the means and SD, respectively.

linked glycoproteins, respectively, were employed in the same experiments. Interestingly, both intercellular and extracellular forms of hPAP21 were sensitive to PNG F as all larger bands were cleaved to similar minor bands of about 26 kDa, indicating that there are N-glycosylation modifications indeed, whatever intercellular or extracellular forms (Fig. 4). However, only intracellular form displayed the sensitivity as endoglycosidase H (Endo H) treatment, whereas the two larger bands, indicated by 'a' and 'b' arrows in Fig. 4, of extracellular forms seem to be resistant to the enzyme although the minor extracellular form was cleaved by Endo H. The data suggested that the glycosylation modifications could be different between intercellular and extracellular forms although all hPAP21 forms were modified through N-glycosylation. Also, the facts, which major forms of extracellular hPAP21 were only sensitive to PNG F, not to Endo H, whereas single intracellular form may be cleaved by both enzymes, implied that, unlike intracellular form with high mannose glycosylation, the N-glycosylation of secreted hPAP21 was mainly synthesized via heterogeneous complex-type oligosaccharide chains, which could take place in Golgi apparatus, not in endoplasmic reticulum [12].

3.4. Effect of N-glycosylation on secretion

To determine whether glycosylation was necessary for hPAP21 secretion, the CHO cells with exogenous hPAP21 were treated for 24 h by tunicamycin, an inhibitor of N-glycosylation. Interestingly, the hPAP21 of CM was not detected by Western blotting assay after tunicamycin treatment, whereas N-glycosylation of the protein in cells was simultaneously inhibited, as the protein band was minished to approximately 26 kDa, the same as the size after PNG F treatment (Fig. 5A), supporting that N-glycosylation was required for the secretion of hPAP21.

Furthermore, to evaluate the effect of the two potential N-glycosylation sites, 121–124 (NDSF) and 171–174 (NVTS), on the secretion of hPAP21, three mutants, Asn121Gln, Asn171Gln and both, were constructed by site-directed mutagenesis assay, respectively, and then the recombinant plasmids containing wild-type and mutants of hPAP21 were transiently transfected into CHO cells. The Western blotting assay showed that the recombinant mutant 2 (Asn171Gln) and mutant 3 (Asn121Gln and Asn171Gln) could not be significantly glycosylated, by contrast, the mutant 1 (Asn121Gln), like wild-type, was glycosylated in extracellular forms (Fig. 5B), suggesting that N-glycosylation of Asn171, not Asn121, was required for the secretory hPAP21. However, it is pointed out that the mutated proteins were still secreted into CM, although the secretion of mutant 2 and mutant 3 was downregulated about 40% as compared with wild-type (Fig. 5C), implying that the N-glycosylation of Asn171 was important, albeit not necessary, for the secretion.

3.5. Secretion pathway of hPAP21

Generally, as known, the secretory protein was transported from ER to Golgi apparatus and *trans*-Golgi network (TGN), and then delivered outside. To determine whether the secretion of hPAP21 did indeed go through Golgi apparatus, the plasmid pEYFP-Golgi (Clontech) containing the membrane-anchoring signal peptide that targets the EYFP fusion protein to the *trans*-medial region of the Golgi apparatus was employed for co-transfection into CHO cells with pcDNA3.1-hPAP21-Myc. The view under the confocal microscope showed that, like growth factor as positive control, hPAP21 and EYFP were co-localized in TGN as the merged images from both proteins emit yellow light (Fig. 6), suggesting that the secretion of hPAP21 was a classical or ER/Golgi-dependent pathway.

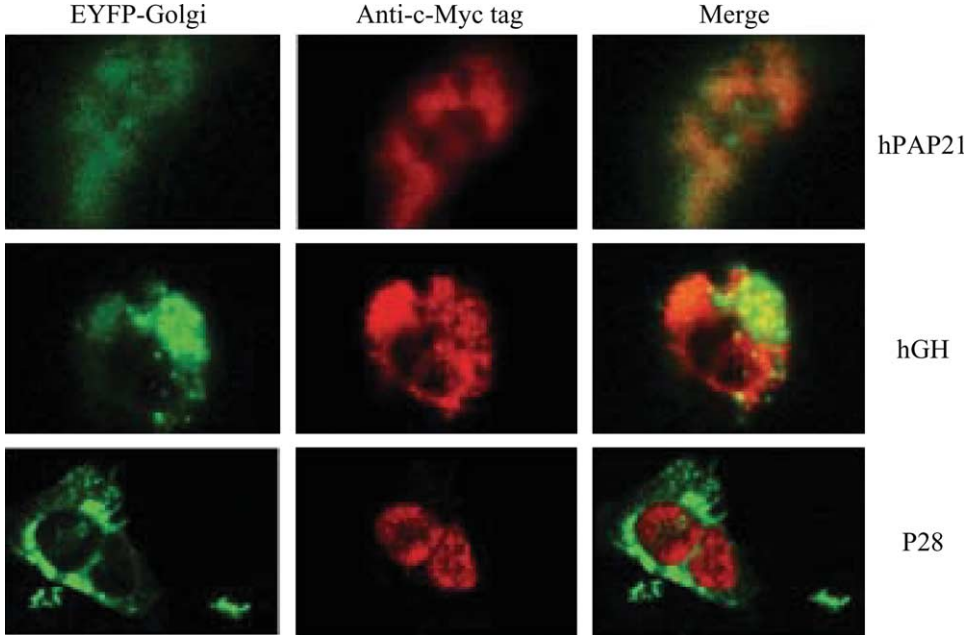

Fig. 6. The subcellular localization of hPAP21. The subcellular localization of hPAP21 in CHO cells was visualized by anti-c-Myc (9E10), together with secondary antibodies conjugated to Cy5 (red), whereas pEYFP-Golgi (green) also was simultaneously co-transfected into the same cells. The merged images showed the co-localized positions (yellow), The slides were observed under a laser-scanning confocal microscope. Human growth hormone (*hGH*) and *p28* genes were used as positive and negative controls, respectively.

4. Discussion

hPAP21, encoded by the hypothetical gene *C2orf7*, was confirmed to be expressed in skeletal muscle, heart and liver by this study albeit the functions of the protein need to be further investigated. Interestingly, hPAP21 has a PA domain in its middle, except for signal peptide in its N-terminus. PA domain exists in many different types of proteases, including the M28 metallopeptidase families that belonged to MH clan [15], although the function of PA domain in these proteases was still largely unknown. Herein, phylogenetic analysis showed that PAP21 was evolutionally conservative as several orthologs from worms to mouse occur, as shown in Fig. 2, implying that the PAP21 might be important for cell behaviors. Recently, the homologs of *Caenorhabditis elegans*, XF509 (T07F12.2) (NP_508831), had been studied by DNA microarray [16] and systematic RNAi functional analyses [17]. However, the knock-down of the gene via RNAi assay did not significantly lead the abnormal phenotype, including morphology, maternal phenotype and embryonic lethality. In this study, we also did not find the effect of hPAP21 on cell cycle and cell proliferation (data not shown).

Interestingly, hPAP21 may be secreted into CM as predicted by many software programs, and the molecular weight of the protein in medium was about 2 kDa bigger than that in the intracellular counterpart. Similar phenomena were observed in mature hTPP I due to the diverse oligosaccharide structures caused by lysosomal glycosidases in lysosomes [18]. Herein, our data indicated that the intracellular form of hPAP21 was mainly modified by high mannose-type glycans, whereas the secreted hPAP21 was primarily modified by complex-type oligosaccharide chains, similar to human α-galactosidase A overexpressed in CHO cells [19,20]. As known, the complex-type oligosaccharide chains were mainly synthesized in Golgi apparatus. We showed here that the intracellular hPAP21 might be partially localized in Golgi apparatus as predicted (Fig. 6).

Segregation is thought to occur in the *trans*-Golgi network (TGN) and is mediated by sorting signals embedded within the protein structure such as N- and O-linked glycans, specific transmembrane domains or glycosylphosphatidylinositol (GPI) anchors, and cytoplasmic domain determinants [21]. N-glycans can mediate protein exit Golgi apparatus to the cell surface in polarized and non-polarized cells [22], playing a structural role or acting as sorting signals as the existence of lectin receptors [23]. Herein, tunicamycin, an inhibitor of N-glycosylation, can markedly decrease the biosynthesis and secretion of hPAP21 into the medium, similar to the effect of tunicamycin on meprin A [10], implying that N-glycans were important for the secretion of hPAP21. Moreover, Asn171 of the protein could be crucial site for N-glycosylation based on the site-directed mutagenesis experiments (Fig. 5). However, hPAP21 without the glycosylation of Asn171 was still secreted into the medium although with less efficiency, suggesting that other unknown glycoproteins or processes were necessary for the secretion of hPAP21, rather than only the N-glycosylation of hPAP21 itself. The N-glycans might prefer a conformational requirement to a sorting signal, by stabilizing the folded forms or improving the interaction with its receptor. Moreover, the complex-type glycans for the secretion of certain glycoproteins would be more efficient than the high mannose type. Such an indirect structural role of N-glycans has been shown in the intracellular transport of VSVG protein [24] and the interaction of CD2 with its receptor [25].

It is estimated that 90% proteins with the sequence Asn-X-Ser/Thr are glycosylated [26], and the glycosylation of Asn-X-Thr sequons is reduced when the sequon is within 60 residues of the C-terminus of the protein [27,28]. However, our data here showed that only the 171–174 aa (NVTS) of hPAP21 was glycosylated, not in the 121–124 aa (NDSF) of the protein, although 171–174 aa (NVTS) was within 18 residues of the C-terminus, implying that the glycosylated efficiency of Asn-Xaa-Thr sequons of hPAP21 could be independent of distance from the C-terminus, similar to membrane dipeptidase [29].

To summarize, we here characterized the expression pattern, secretion and glycosylation of hPAP21, encoded by the hypothetical gene *C2orf7*. Moreover, Endo H and PNG F were employed to evaluate the effect of glycosylation types on secretion of hPAP21. Interestingly, the extracellular forms were primarily sensitive to PNG F, not Endo H, implying that complex N-glycosylation could be required for the secretion of hPAP21. Furthermore, N-glycosylation of Asn171 was confirmed as potential crucial process for the secretory protein via site-directed mutagenesis assay. However, although the data will be helpful to the understanding of molecular functions of hPAP21, we will further investigate the effect of the secretory protein on cell communication and cell behaviors.

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