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# Cellular Basis for Protein C Deficiency Caused by a Single Amino Acid Substitution at Arg15 in the γ-Carboxyglutamic Acid Domain[1]

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**Protein C is a zymogen of an anticoagulant vitamin K-dependent serine protease. Inherited protein C deficiency is often associated with a high risk for venous thromboembolism. It is characteristic of protein C deficiency that most single amino acid replacements result in type I (secretion defect) deficiency. To determine the molecular and cellular bases of protein C deficiency, we expressed recombinant human protein C mutants in which Arg15 was mutated to either Gly, Trp, Gln, Leu, or Pro by a single base exchange. Arg15 is one of the conservative residues in the γ-carboxyglutamic acid (Gla) domains of the vitamin K-dependent coagulation factors, and is also one of the high frequency multiple mutation sites in protein C deficiency. In transient expression studies using human kidney 293 cells, the relative amounts of Arg15 mutants secreted into the medium and determined by enzyme-linked immunosorbent assay (ELISA) were as follows: Gly, 42%; Trp, 14%; Gln, 54%; Leu, 22%; and Pro, 13%, the amount of wild-type (Wt) protein C being taken as 100%. Thus, the order of the secreted amounts of the recombinant mutants was determined to be Wt > Gln > Gly > Leu > Trp, Pro. Pulse-chase experiments using both transiently-transfected and a pool of stably-transfected 293 cells, and stably-transfected BHK cells showed the same order of secretion efficiency. Since this order correlated well with that of the hydrophobicity scale of amino acid side chains, a conformational alteration of the Gla domain resulting in impaired secretion may be dependent on the hydrophobicity of the replaced amino acid. In transient cells, the relative radioactivities of pulse-labeled bands of all recombinant protein C were almost equal, suggesting that the same translational efficiency for Wt and all Arg15 mutants. All of the Arg15-mutated protein C precursors were shown to be located in the same organelle as protein disulfide isomerase (PDI), an endoplasmic reticulum-resident protein, and were sensitive to endoglycosidase H digestion. These results suggest that mutations of the highly conserved Arg15 in the Gla domain of protein C caused a secretion defect to variable degrees depending on replaced amino acid residue.**

**Key words: γ-carboxyglutamic acid domain, inherited deficiency, protein C, secretion, vitamin K-dependent factor.**

Protein C, the 62 kDa zymogen of a vitamin K-dependent serine protease, plays an important role in the regulation of blood coagulation pathways. This protein is activated by the thrombin-thrombomodulin complex, and activated protein C subsequently inactivates factors Va and VIIIa through limited proteolysis in the presence of protein S and phospholipid (*1-3*). Protein C circulates predominantly as a disulfide-linked heterodimer composed of a 21 kDa light chain and a 41 kDa heavy chain (*4, 5*), while 10-15% of protein C circulates in the single chain form without the removal of an internal Lys-Arg dipeptide. Furthermore, about 30% of protein C in the plasma, referred to as β protein C, has a smaller molecular weight due to the absence of a carbohydrate chain at Asn329 (*6*). The light chain consists of a γ-carboxyglutamic acid (Gla) domain containing 9 Gla residues followed by two epidermal growth factor-like domains. The heavy chain is a serine protease domain which is preceded by an activation peptide (*7*). The Gla domain is highly conserved among vitamin K-dependent proteins, including prothrombin, factors VII, IX, and X, and proteins C, S, and Z. This domain is essential for calcium-dependent membrane binding and for the physiological functions of these proteins.

Protein C deficiency is an autosomal dominant inherited disorder. Homozygous or compound heterozygous protein C-deficient individuals with less than 1% protein C activity develop life-threatening purpura fulminans at birth, and heterozygotes are at high risk for thrombophlebitis, deep vein thrombosis and pulmonary embolism (*8*). The prevalence of heterozygous protein C deficiency in the healthy

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Abbreviations: BHK, baby hamster kidney; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; Gla, γ-carboxyglutamic acid; FITC, fluorescein isothiocyanate; PDI, protein disulfide isomerase; Wt, wild type.

population is as high as 1 in 500 (*9*), about 3% of which is associated with a predisposition towards thrombosis (clinically dominant protein C deficiency). Currently, protein C deficiency is classified into two types. Type I deficiency shows parallel reductions in both the enzymatic activity and the antigen level due to the reduced synthesis or secretion of a functional molecule. Type II deficiency shows only a reduction in the functional activity due to the synthesis of an abnormal molecule with reduced specific activity (*8*). Currently, 329 entries from a total of 315 unrelated probands have been reported to the protein C mutation database, most of which were caused by a single amino acid replacement through a single base exchange (*10*). It is characteristic of protein C deficiency that the majority (76%) of these deficiency states have been characterized as heterozygous type I deficiency due to a single amino acid replacement.

Among these mutations, Arg15, one of the conservative residues in the Gla domains of vitamin K-dependent proteins, is known to be a site of high frequency multiple mutations. The replacement of Arg15 (CGG) with Gly (GGG), designated as protein C Yonago, is known as a type II deficiency, and the propositus, who suffered from recurrent thrombosis, had 83% of the antigen and 44% of the anticoagulant activity of protein C (*11, 12*). The mutation of Arg15 to Trp (TGG) has been found in five independent families (*10, 13-15*). The first case was reported as a type I deficiency with a 72% antigen level and 52% activity (*13*), but is now thought to be a type II deficiency (*10*). A second patient with this mutation was reported to have a 90% antigen level and 33% activity, typical of type II deficiency (*14*). A third family showed 41-76% antigen and 31-58% activity levels of protein C, suggesting parallel reduction of the antigen and activity levels (*10, 15*). But, agarose gel electrophoresis followed by Western blotting showed the existence of abnormal as well as normal protein C in the plasma, suggesting type II deficiency (*15*). An Arg15 to Gln (CAG) mutation was reported as type I, as it gave 58% antigen and 60% anticoagulant activity levels (*10, 13, 16*). These clinical data show that the amino acid replacement of Arg15 affects both the secretion and the functional activity of protein C, which is somewhat unusual for mutations at one site.

To clarify these controversial clinical observations, we mutated Arg15 of protein C to five different amino acid residues (Gly, Trp, Gln, Leu, and Pro) by a single base replacement, Gly, Trp, and Gln mutants being naturally occurring, and analyzed the secretion levels and rates of these protein C mutants expressed in human kidney 293 cells and BHK cells, since both types of cells have been well established for the expression of recombinant protein C (*17*).

## MATERIALS AND METHODS

*Materials*—Full-length human protein C cDNA was kindly provided by Dr. Donald C. Foster (ZymoGenetics, Seattle, WA). Purified human plasma protein C and immunoaffinity-purified rabbit anti-human protein C polyclonal IgG were generous gifts from Dr. Walter Kisiel (University of New Mexico, School of Medicine, Albuquerque, NM). Rabbit anti-bovine PDI antiserum was kindly provided by Dr. Masakazu Kikuchi (Ritsumeikan University, Kyoto). Goat anti-human protein C antiserum was purchased from Nordic Immunology (Tilburg, The Netherlands). Rhodamine-labeled sheep anti-rabbit IgG (H+L) and FITC-labeled donkey anti-goat antibody were obtained from The Binding Site (Birmingham, UK). FITC-labeled wheat germ lectin was purchased from Sigma Chemical (St. Louis, MO). Endoglycosidase H was from Boehringer Mannheim Biochemica, and vitamin $K_1$ was the product of Nacalai Tesque (Kyoto). Geneticine disulfate (G418) was purchased from Wako Pure Chemicals (Osaka), and 96-well ELISA plates were obtained from Corning Costar Japan (Tokyo). Other materials were of the highest grade commercially available.

*Site-Directed Mutagenesis and Construction of the Expression Vector*—The scheme for the construction of the expression vector is illustrated in Fig. 1. First, Arg15 in protein C encoded by CGG was mutated to Gly (GGG), Trp (TGG), Gln (CAG), Leu (CTG), or Pro (CCG) by site overlapping mutagenesis using the PCR technique as follows: in a total of 99 $\mu$l, samples comprising 10 $\mu$l of 10× PCR buffer, 200 $\mu$M dNTPs mixture, 100 pmol each of 5′- and 3′-primers, 1 $\mu$g of wild-type (Wt) protein C cDNA in pUC118, and 1 $\mu$l of perfect match polymerase enhancer (Stratagene, La Jolla, CA) were heated at 95°C for 5 min. Then, 1 $\mu$l of *Pfu* DNA polymerase (Stratagene) was added. After overlaying 100 $\mu$l of mineral oil, the reaction was performed for 30 cycles: denaturation at 95°C for 30 s, followed by cooling down to 55°C in 1.5 min for annealing, and polymerization at 75°C for 2.5 min. Mutagenic antisense primer 5′-CACAGATCTCCTCTATGCACTCCC(A/C)CTC-3′ or 5′-CACAGATCTCCTCTATGCACTCC(A/G/T)GCTC-3′ [complementary sequence of nucleotide Nos. 263-290 of protein C cDNA (*18*) with an intrinsic *Bgl*II site (underlined); the mutagenic bases were incorporated as a mixture at the site shown in parentheses] was employed as a 3′-primer, and a universal M13 primer 5′-GTTTTCCCAGTCACGAC-3′ was used as a 5 -sense primer. The PCR products were digested with both *Sph*I and *Bgl*II, and then ligated into the *Sph*I-*Bgl*II region-depleted protein C cDNA in pUC118. Mutations at Arg15 were verified by sequence analyses of the amplified region using a sequence primer, 5′-TGGTCACCGTCGACGTG-3′ [complementary sequence of nucleotide Nos. 353-369]. Then, Wt and five kinds of verified Arg15-mutated protein C cDNAs were ligated into the *Eco*RI site of the pcD2-SR$\alpha$ expression vector equipped with a multicloning site sequence of Bluescript SK. The pcD2 vector was developed by modifying the pcD vector, a cDNA expression cloning vector, by inserting the *neo* transcriptional unit driven by the simian virus 40 (SV40) promoter (*19*). The promoter was further replaced by the SR$\alpha$ promoter; composed of the SV40 early promoter, and the R segment and a part of the U5 sequence of the long terminal repeat of human T-cell leukemia virus type 1. The promoter also contains the pBR322 segment with the $\beta$-lactamase gene ($Amp^r$) and plasmid replication origin (pBR322 ori), as shown in Fig. 1. The expression vectors were purified by CsCl gradient ultracentrifugation.

*Cell Lines and Transfection*—Human kidney 293 cells and BHK cells, obtained from the Japan Research Cell Bank (Tokyo), were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, antibiotic-antimycotic liquid (Gibco BRL

Life Technology) and 10 $\mu$g/ml of vitamin $K_1$. Seven micrograms of expression vector was transfected to $2\times10^5$ cells by the calcium phosphate precipitation method (*19*). For transient expression experiments, medium and cells were harvested after 48 h culture. To obtain a stable transfectant, cells were selected by the addition of 400 $\mu$g/ml G418, and resistant cells grown in 2-3 weeks. A pool of stably-transfected cells was used in the following experiments.

*Enzyme-Linked Immunosorbent Assay (ELISA)*—To measure the concentration of recombinant protein C in culture medium or cell extracts, a sandwich ELISA system was constructed as described (*20, 21*). Briefly, an $F(ab')_2$ fragment was prepared by digestion with pepsin from anti-human protein C IgG. ELISA plates were coated overnight with 200 $\mu$l of 10 $\mu$g/ml $F(ab')_2$ fragment dissolved in 5 mM phosphate buffer, pH 7.5. After washing 3 times with 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.1% Tween 20, an antigen was added. As standards, human plasma protein C serially diluted with DMEM/10% fetal calf serum was used. Samples derived from the culture medium were concentrated 10-fold with a Centricon 30 (Amicon). Cell extracts were solubilized with phosphate-buffered saline containing 1% Triton X-100. After 2 h incubation with standards or samples, 200 $\mu$l of 2,000-fold diluted horseradish peroxidase-conjugated anti-human protein C IgG was added and the preparation was further incubated for 2 h. Finally, immunosorbents were detected by color development, using 150 $\mu$l of 0.4 mg/ml *o*-phenylenediamine in 50 mM sodium acetate, pH 5.0, containing 0.015% (v/v) hydrogen peroxide. After 2-5 min incubation, the reaction was terminated by adding 50 $\mu$l of 3 M sulfuric acid. The absorbance at 490 nm was measured using a Bio-Rad 3550 plate reader.

*Pulse-Chase, Immunoprecipitation, and Gel Electrophoresis*—To determine the secretion rate of Arg15-mutated protein C, pulse-chase experiments were performed as described previously in detail (*21*). Briefly, stably-transfected cells ($5\times10^5$) or transiently-transfected cells were starved for 30 min in DMEM without Met and Cys supplemented with 10% dialyzed fetal calf serum and 10 $\mu$g/ml vitamin K, after which the cells were labeled with 100 $\mu$Ci/ml of EXPRE$^{35}$S$^{35}$S (mixture of 72% L-[$^{35}$S]Met and 18% L-[$^{35}$S]Cys, NEN) for 1 h. After washing, the cells were chased with DMEM supplemented with 10% fetal bovine serum, 2 mM Met, 0.5 mM Cys, and 10 $\mu$g/ml vitamin K. Following incubation for the indicated times, medium was harvested and cells were lysed. The labeled protein C was immunoprecipitated using rabbit anti-human protein C IgG and Staphylosorb (Mercian, Tokyo). The immunoadsorbed proteins were dissociated by heating at 85°C for 5 min and then electrophoresed as described (*22*). Gels were fixed with 50% methanol/10% acetic acid, and immersed in Amplify (Amersham) solution. The radioactivity of the dried gel was measured with a Fujix BAS2000 Bio-Imaging Analyzer system (Fuji Photo Film), and autoradiographs were taken by exposure to Kodak XAR film at −80°C.

*Endoglycosidase H Digestion*—For endoglycosidase H digestion, immunoprecipitated samples were dissolved in 20 $\mu$l of 50 mM sodium citrate buffer, pH 5.5/0.1% SDS and heated at 85°C for 5 min. After cooling, one half of the supernatant was incubated with 1 mU of endoglycosidase H at 37°C for 18 h.

*Immunofluorescence*—To examine the subcellular localization of recombinant protein C mutants in transfected cells, double-label immunofluorescence analysis was performed as described (*21, 23*). Briefly, transiently-transfected 293 cells were fixed with 4% paraformaldehyde in 0.1 M potassium phosphate buffer, pH 7.4. The fixed cells were permeabilized with 0.1% Triton X-100, and then reacted with 1,000-fold diluted rabbit anti-human protein C IgG or goat anti-human protein C serum in phosphate-buffered saline/2% fetal bovine serum. To detect the anti-protein C antibodies, rabbit IgG and goat antiserum were stained with rhodamine-labeled sheep anti-rabbit IgG (H+L) and FITC-labeled donkey anti-goat antibodies, respectively. As organelle markers of the ER and Golgi apparatus, rabbit anti-bovine PDI serum and FITC-labeled wheat germ lectin, respectively, were used. Finally, the cells were mounted in 0.1 M Tris-HCl, pH 8.0/0.1% (w/v) *p*-phenylenediamine/90% (v/v) glycerol, and examined under an Olympus BH2-RFCA microscope. Photographs were taken using Kodak Tri-X pan 400 film.

## RESULTS

*Analysis of the Secretion Efficiency of Arg15 Mutants of Protein C in Transient Cells*—To investigate the effects of Arg15 mutations on the protein C secretion rate, we constructed expression vectors containing cDNAs for Arg15 mutants of protein C, as shown in Fig. 1, and transfected these cDNAs into human kidney 293 cells, as this cell line has been well established for the expression of recombinant protein C (*17*).

Transient expression experiments were performed initially. After 48 h culture, we determined the quantity of recombinant protein C in both cell extracts and conditioned medium by a sandwich ELISA. The mean values of recombinant Wt-protein C in cell extracts and medium were 1.8 and 106 ng/dish, respectively. Taking the amount of Wt as 100%, the relative amount of each Arg15 mutant secreted into the medium was determined to be Gly, 42%; Trp, 14%; Gln, 54%; Leu, 22%; and Pro, 13% (Fig. 2, open bars), giving the order of secretion efficiency of Wt>Gln>Gly>Leu>Trp, Pro. Thus, the mutations of Arg15 to Trp and Pro were shown to result in an about 85% reduction in the secretion level, while those to Gly and Gln gave an about 50% reduction, and the mutation to Leu gave an intermediate value. The relative amounts of Arg15 mutants in the cell extracts were estimated to be Gly, 135%; Trp, 49%; Gln, 108%; Leu, 45%; and Pro, 136%, although the protein C antigen levels determined by ELISA were as low as 0.8-2.4 ng (Fig. 2, closed bars), suggesting that little, if any, intracellular accumulation of these Arg15 mutants of protein C occurred.

Next, the secretion rate of each Arg15 mutant was determined and compared with that of Wt-protein C by pulse-chase analysis using transient 293 cells. The autoradiographic bands of pulse-labeled cell extracts of Wt-protein C and Arg15 mutants exhibited apparently equal intensity (Fig. 3, insert A), suggesting that Arg15 mutations had no effect on the *de novo* synthesis of protein C. After a 2 h chase, secreted recombinant protein C was immunoprecipitated and analyzed by SDS-PAGE (Fig. 3, insert B). Taking the amount of radioactivity of 2 h-chased

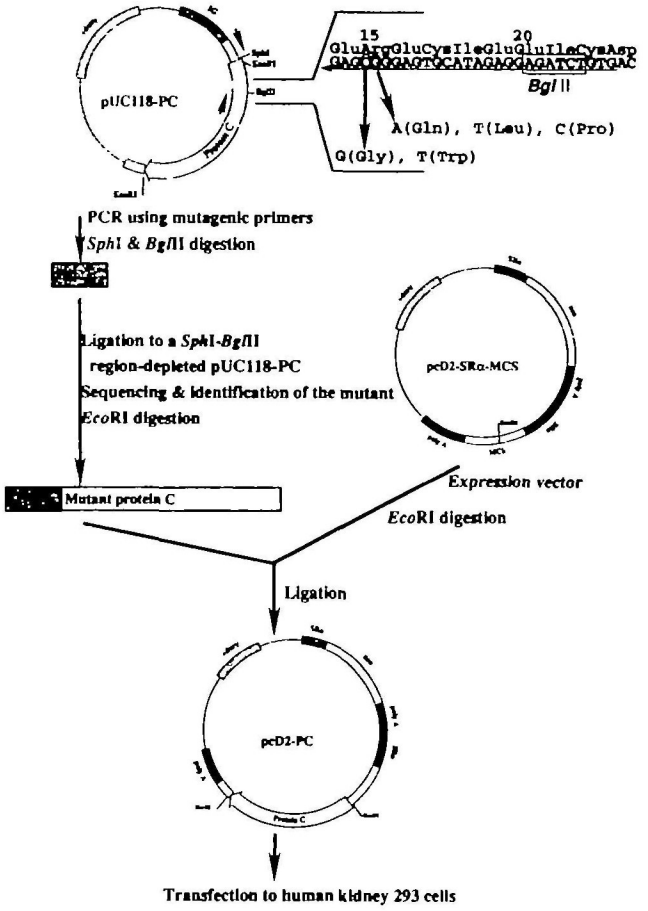


Fig. 1. **The scheme for the construction of the expression vectors of five Arg15 mutants of protein C.** For details, see "MATERIALS AND METHODS."

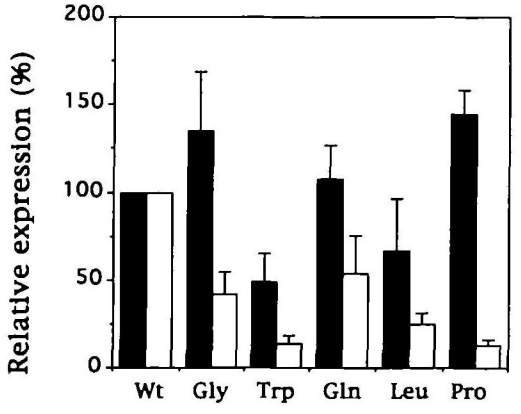


Fig. 2. **Protein C antigen levels in cell extracts and culture medium derived from transiently-transfected 293 cells.** Human protein C cDNA containing Wt or Arg15 mutants was transiently expressed as described under "MATERIALS AND METHODS." After 48 h culture, the antigen levels in cell extracts (closed bars) and culture medium (open bars) were determined by a sandwich ELISA. Each bar shows the mean value of four experiments (12 samples in total).

Wt as 100%, the relative radioactivities of the secreted Arg15 mutants of protein C were determined to be Gly,

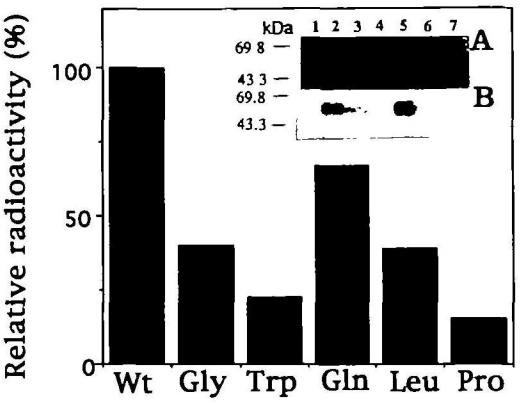


Fig. 3. **Secretion efficiency of Wt and Arg15 mutants of protein C in transient 293 cells.** Transient 293 cells were pulse-labeled for 1 h with 100 $\mu$Ci/ml of EXPRE$^{35}$S$^{35}$S and then chased for 2 h. After immunoprecipitation and SDS-PAGE, the radioactivity in bands corresponding to protein C was quantified with a BAS2000 Bio-Imaging Analyzer. The radioactivity in 2 h-chased medium of Wt-protein C corrected as to the value for pulse-labeled cell extracts was taken as 100%, and relative radioactivities of Arg15 mutants are shown by closed bars. Inserts A and B: SDS-PAGE analyses of cell extracts of 1 h pulse-labeled cell extracts (A), and 2 h-chased conditioned medium (B). Lanes: 1, mock; 2, Wt; 3, Gly; 4, Trp; 5, Gln; 6, Leu; 7, Pro.

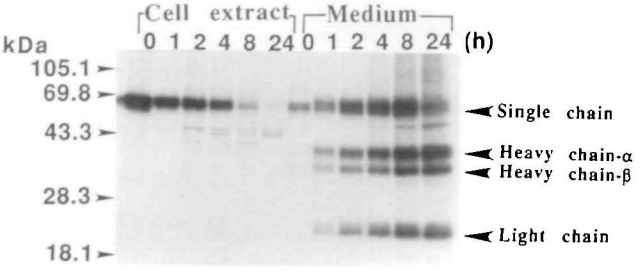


Fig. 4. **Pulse-chase pattern of the Leu-mutant of protein C.** Stable 293 cells expressing the Leu-mutant of protein C were pulse-labeled for 1 h, and then chased for 0, 1, 2, 4, 8, or 24 h. The labeled protein C in cell extracts and conditioned medium was immunoprecipitated as described under "MATERIALS AND METHODS," and then analyzed by SDS-PAGE in the presence of $\beta$-mercaptoethanol.

40%; Trp, 23%; Gln, 67%; Leu, 39%; and Pro, 15% (Fig. 3). The order of the initial secretion rate, Wt>Gln>Gly>Leu>Trp>Pro, was identical to that of the secreted antigen amount with transient 293 cells, as shown in Fig. 2.

*Secretion Rates of Arg15 Protein C Mutants in Stably-Transfected Cells*—To confirm the results of transient expression experiments, we prepared G418-resistant stable 293 cells expressing these mutants. A pool of stable 293 cells that consisted of a sufficient number of clones was used in the subsequent pulse-chase experiments to average the secretion rates of many resistant clones. As an example, the pulse-chase pattern of a stable Leu-mutant is shown in Fig. 4. For cell extracts, a single-chain band corresponding to an apparent molecular mass of 62 kDa was detected, which gradually disappeared with the chase. Concomitant with the decrease of protein C in cell extracts, a two-chain form (heavy- and light-chains) in addition to a single-chain form

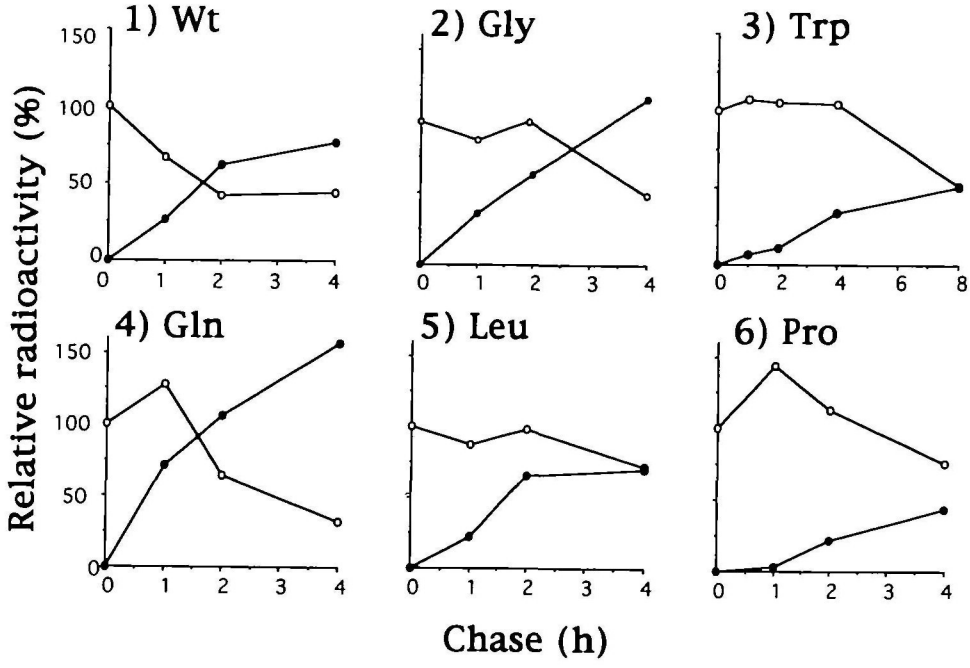


Fig. 5. **Kinetic analyses of the secretion of Wt and Arg15 mutants of protein C.** The relative radioactivities of intracellular and secreted fractions are shown by open and closed circles, respectively, taking the radioactivity values for the cell extracts immediately after pulse-labeling as 100%. Note that the Trp-mutant was chased for 8 h.

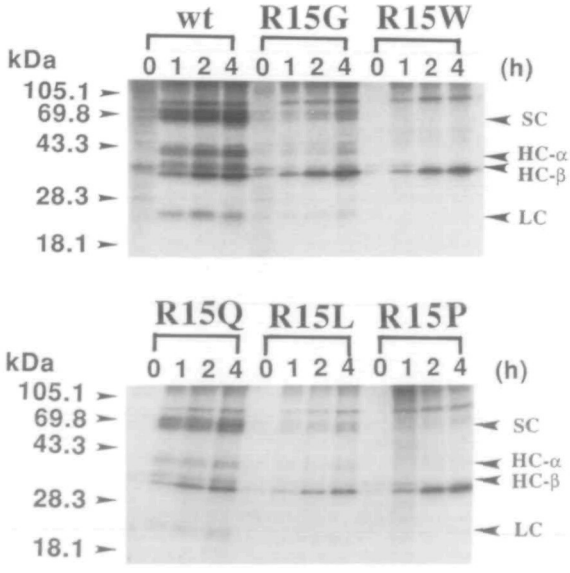


Fig. 6. **Pulse-chase patterns of Wt and Arg15 mutants of protein C in stable BHK cells.** Stable BHK cells were pulse-labeled for 1 h, and then chased for 0, 1, 2, or 4 h. The labeled protein C in the conditioned medium was immunoprecipitated, and analyzed by SDS-PAGE.

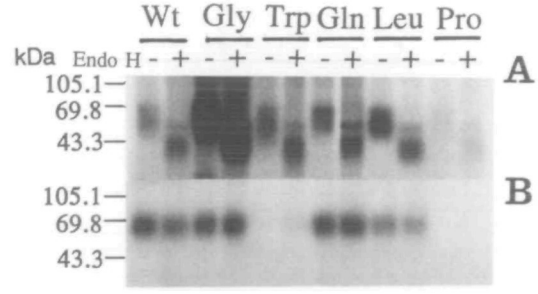


Fig. 7. **Endoglycosidase H digestion of Arg15 mutants of protein C.** A pool of stable 293 cells ($5 \times 10^5$) was pulse-labeled for 1 h and then chased for 4 h. Pulse-labeled cell extracts (panel A) and 4 h-chased conditioned medium (panel B) were immunoprecipitated with anti-human protein C IgG and then subjected to endoglycosidase H digestion, followed by SDS-PAGE.

of protein C increased in the medium. Two types of heavy chain with apparent molecular masses of 41 and 37 kDa were also observed due to the heterogeneity of sugar chains (*6*), and the molecular mass of the light chain was estimated to be 23 kDa. Wt-protein C and all Arg15 mutants showed similar patterns for the mobility of each chain and the degree of conversion into a two-chain form.

To analyze the secretion rates of Arg15 mutants of protein C in stable 293 cells, the radioactivity of each Arg15 mutant was evaluated with a Bio-Imaging analyzer. Quantitative analyses of pulse-chase data for Wt, Gly- and Gln-mutants revealed that the majority of the pulse-labeled radioactivity was recovered from the medium after a 4 h chase (Fig. 5). In contrast, the initial secretion rate of the Leu-mutant was rather low, and those of Pro- and Trp-mutants were much lower. The relative radioactivity of Pro- and Trp-mutants in culture medium, however, reached 100% in 8 and 24 h, respectively, of chase (data not shown), and the total radioactivity remained unchanged, indicating the unlikely intracellular degradation of Arg15 mutants of protein C. The time for 50% disappearance of the radioactivity from cell extracts was estimated to be: Wt, 1.7 h; Gly, 3.9 h; Trp, 7.7 h; Gln, 2.8 h; Leu, 5.4 h; and Pro, 6.3 h, yielding the order of secretion efficiency, Wt > Gln > Gly > Leu > Pro > Trp, which is consistent with the transient expression data shown in Figs. 2 and 3.

To examine the cell line specificity, we also performed pulse-chase experiments with a pool of stable BHK cells. Figure 6 shows the SDS-PAGE analysis of immunoprecipitates derived from the secreted fraction of each Arg15 mutant. The secretion rates of Trp- and Pro-mutants were

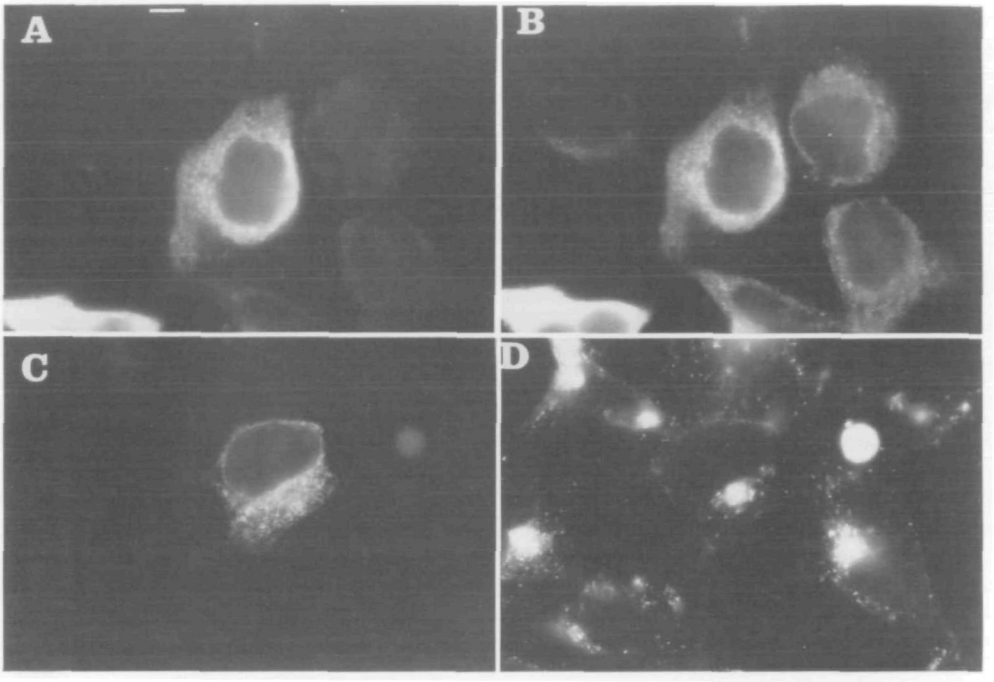


Fig. 8. **Immunofluorescent localization of the Pro-mutant of protein C.** Human kidney 293 cells were transiently-transfected with the pcD2 expression vector containing Arg15→Pro mutated protein C cDNA. Two days after transfection, the cells were fixed, permeabilized, and then subjected to immunofluorescence staining. Panels A and B were stained with goat anti-human protein C antiserum and rabbit anti-bovine PDI antiserum, respectively, each followed by with FITC-labeled donkey anti-goat IgG and rhodamine-labeled sheep anti-rabbit IgG, respectively. Panel C was first stained with rabbit anti-human protein C IgG and then with rhodamine-labeled sheep anti-rabbit IgG, and Panel D is FITC-labeled wheat germ lectin. Panels A and C show the immunofluorescence of protein C. Panels B and D show the staining patterns of PDI and FITC-wheat germ lectin, respectively. Scale bar at the top of Panel A: 10 $\mu$m.

significantly lower than that of Wt, suggesting that there is no apparent difference between human and hamster kidney cells in the secretion rates of Wt and Arg15 mutants of protein C.

*Subcellular Localization of Arg15 Mutants*—Finally, to determine the distribution of each Arg15 mutant of protein C in cells, we performed endoglycosidase H digestion of the intracellular and secreted protein C. After endoglycosidase H digestion, all bands of intracellular protein C shifted to lower molecular mass (by 10 kDa) positions, as shown on SDS-PAGE (Fig. 7A). In contrast, those of secreted protein C were resistant to the enzyme (Fig. 7B). These data suggest that intracellular protein C has carbohydrate chains of high mannose-type and is localized in the ER to *cis*-Golgi compartment.

To further localize the subcellular site for protein C mutants, we performed immunofluorescence staining of 293 cells, transiently expressing the Pro-mutant (Fig. 8). All Pro-mutant molecules were detected in the reticular organelle throughout the cytoplasm but not in the nucleus (Fig. 8, A and C). These patterns were identical with that of PDI, the ER-resident protein (Fig. 8B), but not with that of FITC-wheat germ lectin, a Golgi marker (Fig. 8D). Wt and other Arg15 mutants of protein C showed similar immunofluorescence staining patterns (data not shown). These results suggest that all Arg15 mutants of the protein C precursor, regardless of the replaced amino acid residue, are predominantly localized in the ER.

## DISCUSSION

Protein C deficiency is an autosomally inherited disorder that is associated with an increased risk of venous thrombosis. Currently, more than 300 mutations have been identified throughout the molecule, and the majority of protein C deficiency is classified into the heterozygous type I category (*10*). In general, type I deficiency is caused by mutations such as a deletion or introduction of an appropriate splice site, deletion of a portion of the coding sequence or insertion of a stop codon in the coding sequence. In protein C deficiency, however, most single amino acid replacements cause type I deficiency. At present, the cellular mechanism of protein C deficiency is poorly understood. Among a total of 132 different single nucleotide substitutions, 42 (32%) occur in CpG dinucleotides; *i.e.* either C→T or G→A transitions compatible with a model of methylation-mediated deamination, and 19 different C→T or G→A transitions in a total of 16 different CpG dinucleotides account for the pathological lesions in 41% of patients (*10*). Indeed, in the case of Arg15, three types of mutation, Arg15 (CGG)→Gly (GGG), Trp (TGG), and Gln (CAG), have been found in thrombotic patients, the protein C antigen level decreasing variably to 55 to 90% of normal (*10-16*). Since the antigen level of a heterozygously deficient patient implies impaired secretion or instability of the mutant molecule, in this study we examined the secretion mechanism of Arg15 protein C mutants using cell biological techniques. First, we mutated Arg15 to Gly, Trp, Gln, Leu, or Pro by a single base replacement, three of which (Gly, Trp, and Gln) are naturally occurring, and found that mutations of Arg15 caused decreases in the secretion level and rate of protein C in the order of Wt > Gly, Gln > Leu > Trp, Pro, in both transiently-transfected and stably-transfected 293 cells (Figs. 2, 3, and 5). This was confirmed by a co-expression experiment on protein C mutants with antithrombin as a control in transient 293 cells, showing that Trp and Pro mutants resulted in greater decreases in the secretion level and rate than Wt, or Gly, Gln or Leu mutant (data not shown). These results clearly demonstrate that the secretion efficiency of Arg15 mutants of protein C from 293 cells is dependent on the replaced amino acid residue. Clinical observations showed that, in

patients with a heterozygous Arg15→Trp mutation, the antigen levels varied in the range of 55-90% of normal, but our cellular analyses showed that the Trp mutation resulted in a rather severe secretion defect, giving an antigen level of 14% of normal. Arg15 to Gly and Gln mutants of protein C were relatively secretable in cellular experiments, whereas clinically only a Gly mutant has been reported to be secreted normally into the circulation.

Several explanations are possible for the discrepancies between clinical data and experimental results obtained for the Arg15 mutants described above. First, we used kidney cell lines, not liver cells in which protein C is biosynthesized. Since liver cell lines, such as HepG2 cells, secrete protein C endogenously (*6*), it is difficult to distinguish endogenous protein C and its recombinant introduced by the transfection. BHK and 293 cells have been well characterized as to protein C secretion and the levels of post-translational modifications, and these cell lines are thought to be appropriate tools for investigating the secretion mechanisms of vitamin K-dependent factors (*17*). The second possibility for the discrepancies between clinical data and our results is the transfection efficiency. Actually, Guarna *et al.* reported that the rate of secretion of protein C by BHK cells was increased by increasing the cDNA copy number per cell, but higher transfection (>240 cDNA copy/cell) resulted in a decrease of the secretion efficiency (*24*). Therefore, to average the transfection efficiency, we employed a neomycin-resistant vector, not an inducible vector like they used. Moreover, we used a pool of stable cells, not an isolated clone, to average the transfection efficiency of a sufficient number of clones. As a result, we obtained apparently equal intensities of pulse-labeled Wt and Arg15 mutants of protein C (Fig. 3), suggesting the same translational efficiency. Thus, the difference in the secreted amount of Arg15 mutants may be attributed to the difference in secretion rate. The third possibility for the discrepancies between clinical data and our results is the rate of clearance. Protein C, as well as factor VII, shows a relatively more rapid clearance rate in plasma (half-life = 6-8 h) than factor IX, factor X and prothrombin (with half-lives of approximately 1, 2, and 3-4 d, respectively) (*25*). This suggests that protein C and factor VII are relatively unstable in plasma, and might be involved in an accelerated clearance mechanism. Although we have investigated the aspect of secretion in this study, it is highly possible that each Arg15 mutant of protein C shows a different clearance rate, which may account for the discrepancies.

Arg15 in the Gla domain is highly conserved among vitamin K-dependent proteins, including prothrombin, factors VII, IX, and X, and proteins C and S derived from many species. Zhang *et al.* extensively studied the importance of Arg15 in protein C by expressing an Arg15→Leu mutant in 293 cells (*26-28*). The recombinant Leu-mutant showed only 19% anticoagulant activity, and no fluorescence alteration occurred upon the addition of $Ca^{2+}$, indicating that Arg15 plays an important role in anticoagulant activity and in maintenance of the $Ca^{2+}$-dependent structure of protein C. Based on the crystal structure of bovine prothrombin fragment 1, the HH21 proton of Arg15 of protein C (Arg16 in prothrombin) was shown to be sufficiently close (0.23 nm) to the oxygen of the OE4 group of Gla16 (Gla17 in prothrombin) to form a hydrogen bond, which stabilizes the conformational state optimal for activity (*26*). Since Arg15 is important for the conformation of the Gla domain and interacts with internal hydrophobic residues, the mutation may affect protein folding, resulting in a secretion defect. The mutation of Arg15 to Trp or Pro may alter the tertiary structure of the Gla domain drastically by virtue of its side chain, although mutation of Arg15 to Gly or Gln may not affect it so much, and the mutation to Leu may result in intermediate alteration. Thus, these effects may be reflected by the different secretion level of each Arg15 mutant of protein C. Indeed, the hydrophobicity scale of amino acid side chains calculated from the free energy values ($\Delta f_t$) of transfer from aqueous to organic solutions were shown to be: Gly, 0 kcal/mol; Trp, 3.4 kcal/mol; Gln, −0.1 kcal/mol; Leu, 1.8 kcal/mol; and Pro, 2.6 kcal/mol (*29*). The order of the hydrophobicity, Gln < Gly < Leu < Pro < Trp, thus correlated well with that of secretion efficiency. Besides Arg15 mutations, Arg9→Cys, Ser12→Cys, Glu20→Ala, Glu25→Gln, Glu25→Lys, Glu26→Lys, and Val34→Met mutations have been reported for the Gla domain of protein C, Arg15 mutations and Glu25→Lys being exceptional, where most of them are thought to lead to type II deficiency (*10*). Although the majority of protein C deficiencies caused by an amino acid replacement have been characterized as type I deficiency, some protein C deficiencies may involve both impaired secretion and dysfunctional molecules, similar to the pleiotropic effects-type mutation in antithrombin that shows impaired interactions with thrombin and heparin, coupled with a reduced plasma concentration (*30*).

The cellular basis of type I deficiency has been studied for $\alpha_2$-plasmin inhibitor and in more detail for $\alpha_1$-protease inhibitor. $\alpha_2$-Plasmin inhibitor deficiency is a secretion defect caused by a deletion of Glu137 ($\alpha_2$-plasmin inhibitor Okinawa) (*31*). Pulse-chase and ELISA experiments using transient COS cells showed that a replacement of Glu137 with an amino acid other than Cys had little effect on secretion, while a deletion of a single amino acid in the 127 to 137 region severely impaired the secretion (*32*), suggesting that the structural integrity of the region, rather than the specific amino acid residue, is important for the intracellular transport and secretion. In cases of Z-variants of $\alpha_1$-protease inhibitor that are due to either a Val213→Ala or Glu342→Lys mutation, the amino acid substitution at position 342 was shown to be critical, leading to a defect in secretion and degradation in the ER (*33*). In a case of protein C deficiency, protein C-Nagoya, shown to be caused by a deletion of a single guanine in the protein C gene resulting in a frameshift and elongation of the COOH-terminal 42 residues, was studied as to the cellular basis of its deficiency using stable $\psi$-2 cells. Pulse-chase analyses of Wt and Nagoya-type protein C suggested impaired secretion of protein C Nagoya (*34*). Unlike $\alpha_2$-plasmin inhibitor Okinawa or Z-variants of $\alpha_1$-protease inhibitor, Arg15 mutants of protein C showed an amino acid-dependent influence on the secretion.

All Arg15 mutants of protein C studied here, as well as Wt, were predominantly localized in the ER (Figs. 7 and 8). Recently, we found that warfarin, an antagonist of vitamin K, causes degradation of the protein C precursor in the ER through the quality control mechanism, suggesting that the incomplete synthesis of Gla residues resulted in the intracellular degradation (*21*). In the presence of warfarin, the

molecular mass of the light chain was reduced from 23 to 20.5 kDa in a dose-dependent manner, indicating the lack of Gla formation. In the present study, we could not find a low molecular form of the light chain in any of the Arg15 mutants examined, suggesting that Gla formations were apparently normal. Indeed, a Leu-mutant, as well as recombinant Wt protein C expressed in 293 cells, was shown to be fully $\gamma$-carboxylated with 9 Gla residues (*26*). Therefore, not only Gla formation but also proper folding of the polypeptide are required for the transport of protein C from the ER to the Golgi apparatus. Hammond *et al.* (*35*) proposed a model for folding and quality control in the ER. In their model, there are four main stages: (i) stepwise trimming of glucose of *N*-linked oligosaccharides, (ii) binding of calnexin, a membrane-bound ER chaperone, to proteins with monoglucosidated sugar chains, (iii) reglucosylation of glucose-free sugar chains on incompletely folded glycopeptide chains, and (iv) release of folded chains from the re- and deglucosylation cycle (*35*). Therefore, secretion defects are likely to result from retardation in the ER of formation of the proper conformation for secretion by the quality control system, and the observed differences in the secretion rate caused by the type of replaced amino acid residue may be due to the differences in the association time with molecular chaperones, such as calnexin, in the ER. Furthermore, ER-associated degradation of a improperly folded polypeptide has been reported (*36*). In the case of warfarin-treated protein C, a dramatic decrease in the total radioactivity which was not inhibited by the addition of breledin A, an ER-Golgi transport inhibitor, was observed, suggesting ER-associated degradation of under-$\gamma$-carboxylated protein C (*21*). Although such a dramatic decrease in radioactivity was not observed for Arg15 mutants of protein C, Trp- and Pro-mutants may be partially degraded intracellularly. Indeed, recently, we found that a part of a Pro407→Leu mutant of antithrombin (antithrombin Utah) is degraded intracellularly when expressed in BHK cells [Tokunaga, F., Shirotani, H., and Koide, T. (submitted)]. To further investigate the secretion mechanism of protein C and quality control in the ER, studies are in progress to identify the molecular chaperones associated with the Arg15 mutants of protein C.

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