Warfarin Causes the Degradation of Protein C Precursor in the Endoplasmic Reticulum†

Fuminori Tokunaga, Sadao Wakabayashi, and Takehiko Koide*

Department of Life Science, Faculty of Science, Himeji Institute of Technology, Harima Science Park City, Hyogo 678-12, Japan

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ABSTRACT: Warfarin, an antagonist of vitamin K, is known to disrupt the microsomal vitamin K cycle, which results in a decrease in the plasma level of protein C, an anticoagulant factor, as well as some other vitamin K-dependent coagulation factors. Here, we examined the effect of warfarin on the secretion of recombinant protein C expressed in human kidney 293 or BHK cells. In transient expression, warfarin caused a 2−4-fold decrease in the quantity of protein C secreted, compared to findings with vitamin K-treated cells. Pulse−chase experiments using stable cells showed that, although recombinant protein C was secreted in the presence of vitamin K, the decrease in the total amount of radioactivity in the warfarin-treated cells suggested intracellular degradation. This degradation depended on the concentration of warfarin and was not inhibited by an endoplasmic reticulum (ER)−Golgi transport inhibitor (brefeldin A) or by lysosomotropic inhibitors (chloroquine and NH_4Cl). Thus, protein C synthesized in the presence of warfarin is probably selectively degraded, and this degradation occurs in a pre-Golgi, nonlysosomal compartment. Among the protease inhibitors tested, *N*-α-acetyl-Leu-Leu-methioninal and *N*-α-acetyl-Leu-Leu-norleucinal blocked the degradation of protein C precursor synthesized in the presence of warfarin, and the precursor accumulated intracellularly, in a dose-dependent manner. Both inhibitors, however, did not disturb the secretion of protein C precursor in the vitamin K-treated cells. Thus, a cysteine protease(s) appeared to be responsible for the degradation. Moreover, protein C synthesized in the presence of warfarin was located in the same organelle as protein disulfide isomerase, an ER-resident protein, and the intracellular protein C was sensitive to endoglycosidase H digestion. This evidence suggests that protein C synthesized in the presence of warfarin was selectively degraded by a cysteine protease(s) in the ER through a "quality control" mechanism.

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 the activated protein C inactivates factors Va and VIIIa with limited proteolysis in the presence of protein S and phospholipid (Esmon, 1987, 1989). Protein C circulates predominantly as a disulfide-linked heterodimer composed of a 21 kDa light chain and a 41 kDa heavy chain (Kisiel et al., 1976; Stenflo, 1976), and 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 plasma, referred to as β-protein C, has a molecular weight smaller than that of the predominant form, because of the lack of a carbohydrate chain at Asn329 (Miletich & Broze, 1990). The light chain consists of a γ-carboxyglutamic acid (Gla)[1] domain containing nine Gla residues and two epidermal growth factor-like domains. The heavy chain is a serine protease domain preceded by 12 amino acid residues of an activation peptide (Foster et al., 1985). The Gla domain is highly conserved among vitamin K-dependent proteins, including prothrombin, factors VII, IX, and X, and proteins C, S, and Z. The domain is essential for calcium-dependent membrane binding and for expression of the physiological function of these proteins.

Warfarin, an antagonist of vitamin K, is a widely prescribed and potent coumarin-type oral anticoagulant. This antagonist strongly inhibits the enzymatic activities of vitamin K epoxide reductase and vitamin K quinone reductase (Suttie, 1985). This metabolic disruption of the vitamin K cycle results in decreased availability of vitamin K hydroquinone, an active cofactor form of vitamin K, for microsomal vitamin K-dependent carboxylase, which eventually results in a decreased synthesis of Gla residues in vitamin K-dependent coagulation factors. Pharmacological alteration of vitamin K metabolism by warfarin has been well established in anticoagulant therapy. Also, warfarin is known to cause a significant decrease in the plasma concentrations of vitamin K-dependent proteins (Thompson, 1977; Bertina

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* Address correspondence to Dr. Takehiko Koide, Department of Life Science, Faculty of Science, Himeji Institute of Technology, Harima Science Park City, Hyogo 678-12, Japan. Telephone: +81-7915-80212. FAX: +81-7915-80210. e-mail: koide@sci.himeji-tech.ac.jp.

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[1] Abbreviations: ALLM, *N*-α-acetyl-Leu-Leu-methioninal; ALLN, *N*-α-acetyl-Leu-Leu-norleucinal; BHK, baby hamster kidney; DCI, 3,4-dichloroisocoumarin; DMEM, Dulbecco's Modified Eagle's medium; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; Gla, γ-carboxyglutamic acid; PDI, protein disulfide isomerase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLCK, *N*-α-tosyl-L-lysyl chloromethyl ketone; TPCK, *N*-α-tosyl-L-phenylalanyl chloromethyl ketone.

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et al., 1982; Zivelin et al., 1993). Protein C in normal human plasma circulates with a mean value of 4.0 μg/mL (65 nM). After warfarin administration, however, total protein C antigen decreases to 53% of the normal level (Miletich & Broze, 1987), and sometimes there is a thrombotic complication, primarily, coumarin-induced skin necrosis (Broekmans & Conard, 1988). The pathogenesis of this phenomenon may be explained by a rapid drop in the protein C and factor VII levels (both with a half-life time of 6−8 h), in contrast to the delayed drop in factors IX and X and prothrombin (Viganò et al., 1984).

To examine the effect of warfarin on the biosynthesis and secretion of vitamin K-dependent proteins, we analyzed the level and rate of secretion of recombinant human protein C expressed in human kidney 293 cells or BHK cells, since both species have been well established for the expression of recombinant protein C, and the levels of secretion of protein C from these cells are enhanced in the presence of vitamin K (Foster et al., 1990; Grinnell et al., 1990; McClure et al., 1992). We have now obtained evidence that warfarin treatment of both cell lines caused a decrease in both the rate of secretion and the quantity of secreted protein C, due to intracellular degradation. As this degradation occurred in a pre-Golgi, nonlysosomal compartment, it is likely that a "quality control" mechanism in the endoplasmic reticulum (ER) degrades protein C precursor synthesized in the presence of warfarin.

MATERIALS AND METHODS

Materials. 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 protein disulfide isomerase (PDI) serum was kindly provided by Dr. Masakazu Kikuchi (Protein Engineering Research Institute, Osaka, Japan). Goat anti-human protein C serum 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). Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin], brefeldin A, diamide (diazinedicarboxylic acid bis(*N*,*N*-dimethylamide)), chloroquine, *N*-α-acetyl-Leu-Leu-methioninal (ALLM), *N*-α-acetyl-Leu-Leu-norleucinal (ALLN), FITC-labeled wheat germ lectin, and chymostatin were purchased from Sigma Chemical (St. Louis, MO). Amastatin, antipain, E-64 [[(L-3-*trans*-carboxyoxiran-2-yl)-carbonyl]-L-Leu-agmatin], elastinal, leupeptin, and phosphoramidon were the products of Peptide Institute (Osaka, Japan). 3,4-Dichloroisocoumarin (DCI) and endoglycosidase H were obtained from Boehringer Mannheim Biochemica. Vitamin K_1 and *N*-α-tosyl-L-lysyl chloromethyl ketone (TLCK) were from Nacalai Tesque (Kyoto), *N*-α-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) was from Aldrich, and geneticine disulfate (G418), diethyl maleate, and 3-amino-1,2,4-triazole were purchased from Wako Pure Chemicals (Osaka, Japan).

Construction of the Expression Vector and Transfection to Cells. 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 (Chen & Okayama, 1987). The promoter

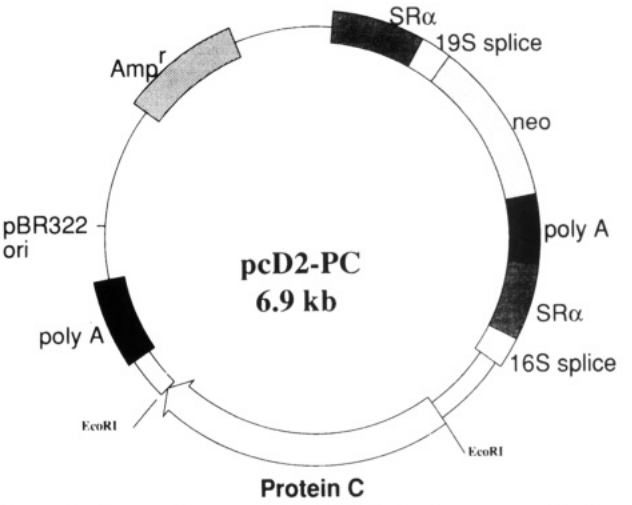

FIGURE 1: Expression vector (pcD2-PC) for human protein C.

was further replaced by SRα promoter, which is 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 (Takebe et al., 1988). It also has the pBR322 segment with the β-lactamase gene (Amp^r) and the plasmid replication origin (pBR322 ori). Full-length human protein C cDNA, kindly provided by Dr. Donald C. Foster (ZymoGenetics Inc., Seattle, WA), was ligated to the *Eco*RI site of the pcD2-SRα expression vector equipped with a multicloning site sequence of Bluescript SK, as illustrated in Figure 1. The expression vector, pcD2-PC, thus constructed was purified by CsCl gradient ultracentrifugation. Human kidney 293 and BHK-21 (C-13) cells, obtained from Japan Research Cell Bank (Tokyo), were maintained in Dulbecco's Modified Eagle's medium (DMEM)/10% fetal calf serum/glutamine/antibiotic−antimycotic liquid (Gibco BRL Life Technology)/10 μg/mL (22.2 μM) vitamin K or 1 μM warfarin. Seven micrograms of expression vector, pcD2-PC, was transfected in 2×10^5 cells by the calcium phosphate coprecipitation method (Chen & Okayama, 1987). For transient expression experiments, medium and cells were harvested after 48 h of culture. To obtian a stable transfectant, cells were selected by the addition of 400 μg/mL G418. The resistant cells were fully grown in 2−3 weeks. A pool of stable 293 cells and a selected stable BHK clone, which produced a high level of protein C, were used.

Enzyme-Linked Immunosorbent Assay (ELISA). To measure the concentration of recombinant protein C in culture medium or cell extracts, an ELISA system was constructed as described (Boyer et al., 1984). Briefly, a $F(ab')_2$ fragment was prepared by digestion with pepsin from anti-human protein C IgG. ELISA plates were coated overnight with 200 μL of 10 μg/mL $F(ab')_2$ fragment dissolved in 5 mM phosphate buffer (pH 7.5). After the plates were washed 3 times with 50 mM Tris-HCl (pH 7.5)/0.15 M NaCl/0.1% Tween 20, an antigen was added. For standards, human plasma protein C serially diluted with DMEM/10% fetal calf serum was used. Samples derived from the culture medium of 293 cells were concentrated 10-fold by Centricon 30 (Amicon), and those of BHK cells were used without concentration. Cell extracts were solubilized with phosphate-buffered saline containing 1% Triton X-100. After a 2 h incubation with standards or samples, 200 μL of 2000-fold diluted horseradish peroxidase-conjugated anti-human protein

C IgG, prepared according to the method of Nakane and Kawaoi (1974), was added, and the preparation was further incubated for 2 h. Finally, immunosorbents were detected by color development, using 150 μL 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 of incubation, the reaction was terminated by adding 50 μL 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 examine the rate of secretion of recombinant protein C in the presence of vitamin K or warfarin, pulse−chase experiments were done. Stable cells (5×10^5 293 cells or 2.5×10^5 BHK cells), plated on the previous day in 35 mm dishes, were starved for 30 min with DMEM without Met and Cys (Sigma)/10% dialyzed fetal calf serum/10 μg/mL vitamin K or warfarin, and then the cells were labeled with 100 μCi/mL EXPRE^{35}S^{35}S (mixture of 72% L-[^{35}S]Met and 18% L-[^{35}S]Cys, NEN) for 1 h. After the cells were washed in phosphate-buffered saline, they were chased with DMEM/10% fetal calf serum/2 mM Met/0.5 mM Cys/vitamin K or 1 μM warfarin. Following incubation for the indicated times, medium was harvested and cells were lysed in 300 μL of 10 mM Tris-HCl (pH 7.4)/0.1% SDS/1% Nonidet P-40/0.15 M NaCl/10 mM Met/2 mM EDTA/0.1 mM phenylmethanesulfonyl fluoride/1 μg/mL leupeptin/1 μg/mL aprotinin. The labeled protein C was immunoprecipitated using rabbit anti-human protein C IgG and Staphylosorb (Mercian, Tokyo). After they were washed 3 times with 10 mM Tris-HCl (pH 7.4)/0.1% SDS/0.1% Nonidet P-40/10 mM Met/2 mM EDTA, immunoabsorbed proteins were dissociated by heating at 85 °C for 5 min and then electrophoresed, as described (Laemmli, 1970). Gels were fixed with 50% methanol/10% acetic acid for 30 min and then immersed in Amplify (Amersham) solution for 30 min. The radioactivity of dried gels was measured using a Fujix BAS2000 Bio-Imaging Analyzer system (Fuji Photo Film), which can analyze radioactivity quantitatively and sensitively on the basis of photostimulated luminescence (Amemiya & Miyahara, 1988). Autoradiographs were taken by exposure to Kodak XAR films at −80 °C.

Endoglycosidase H Digestion. For endoglycosidase H digestion, immunoprecipitated samples were dissolved in 20 μL of 50 mM sodium citrate buffer (pH 5.5)/0.1% SDS and heated at 85 °C for 5 min. After cooling, half of the supernatant was incubated with 1 munit of endoglycosidase H at 37 °C for 18 h.

Immunofluorescence. To examine the subcellular localization of recombinant protein C in transfected cells, a double-label immunofluorescence analysis was made as described (Osumi et al., 1991). Briefly, transiently transfected 293 cells were fixed with 0.1 M potassium phosphate buffer (pH 7.4)/4% paraformaldehyde. The fixed cells were permeabilized with 0.1% Triton X-100 and then reacted with 1000-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 antibody, respectively. For organelle markers of ER and Golgi apparatus, rabbit anti-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-Xpan 400 films.

Table 1: Protein C Antigen Levels in Cell Extracts and in Culture Medium Derived from Transiently Transfected 293 or BHK Cells Cultured in the Presence of 10 μg/mL Vitamin K or 1 μM Warfarin[a]

		protein C antigen (ng/dish) (mean ± SD, $n = 4$)	
cells		vitamin K	warfarin
293	cell extracts	1.8 ± 0.8	4.2 ± 0.6
	culture medium	344 ± 13	91 ± 7
BHK	cell extracts	<0.9	<0.9
	culture medium	607 ± 154	321 ± 35

[a] Human protein C cDNA was transiently transfected as described in the Mateials and Methods section. After 48 h of culture, the antigen levels in cell extracts and in culture medium were measured by sandwich ELISA.

RESULTS

Effect of Warfarin on the Secretion of Recombinant Protein C from Mammalian Cell Lines. To investigate the effect of warfarin on the secretion of recombinant human protein C, we constructed the expression vector, pcD2-PC, as shown in Figure 1, and expressed it in human kidney 293 cells or BHK cells, since both cell lines have been well established for the expression of recombinant protein C (Foster et al., 1990; Grinnell et al., 1990; McClure et al., 1992).

Transient expression experiments were done first. After a 48 h culture in the presence of 10 μg/mL (22.2 μM) vitamin K or 1 μM warfarin, we determined the quantity of recombinant protein C both in cell extracts and in conditioned medium by sandwich ELISA. In vitamin K-treated 293 cells, mean values of protein C expressed in the cell extracts and the medium were determined to be 1.8 and 344 ng/dish, respectively (Table 1). In the presence of warfarin, protein C antigens in the cell extracts and in the medium were 4.2 and 91 ng/dish, respectively. Thus, warfarin treatment induced a 2.3-fold accumulation within cells, whereas only 26% as much protein C was secreted compared to the value for vitamin K-treated 293 cells. A similar degree of intracellular accumulation and a reduced level of secretion in the presence of warfarin were observed with HepG2 cells (unpublished results). In BHK cells, warfarin treatment also decreased the quantity of secreted protein C to 53% of that for vitamin K-treated cells. Since the protein C antigen levels of cell extracts obtianed from either cells in the presence of vitamin K or warfarin were below the level of detection, it is not likely that warfarin-induced intracellular accumulation occurs in BHK cells.

Next, the level of secretion of recombinant protein C in the presence of various concentrations of warfarin was determined by pulse−chase analysis using stable BHK cells. The relative radioactivities of pulse-labeled bands were in the range of 84−106% of that of vitamin K-treated cells, suggesting that warfarin had no effect on the *de nono* synthesis of protein C (Figure 2, insert A). The molecular masses of pulse-labeled intracellular protein C derived from vitamin K- or warfarin-treated BHK cells were estimated as 59 and 54 kDa, respectively (Figure 2, insert A, and see Figure 5, lanes 1 and 3). Thus, the molecular mass of

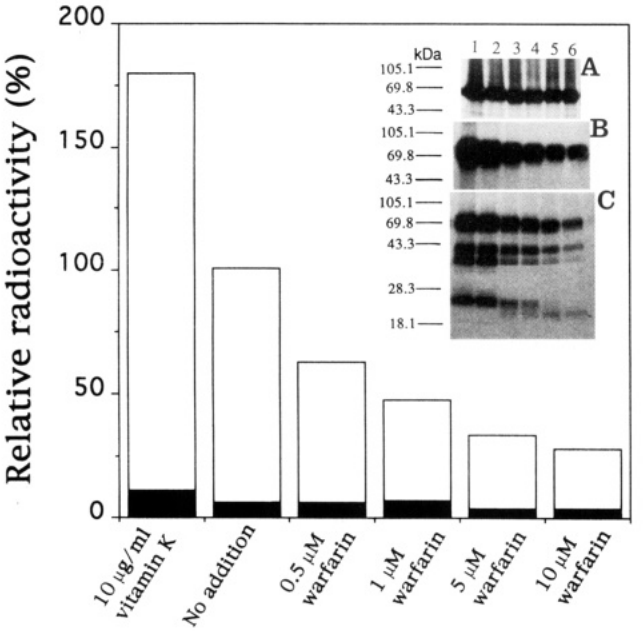

FIGURE 2: Secretion efficiency of protein C in the presence of vitamin K or various concentrations of warfarin in stable BHK cells. Stable BHK cells were pulse-labeled for 1 h with 100 μCi/mL EXPRE^{35}S^{35}S (a mixture of [^{35}S]Met and [^{35}S]Cys) and chased for 8 h in the presence of 10 μg/mL vitamin K, various concentrations of warfarin, or without any addition to the medium. After immunoprecipitation and SDS−PAGE, the amount of radioactivity in bands corresponding to protein C was quantified using a BAS2000 Bio-Imaging Analyzer. The sum of radioactivity obtained from 8 h chased medium and cell extracts (of control cells referred to as no addition) was taken as 100%, and the relative radioactivities of cell extracts and medium obtained from cells cultured under each condition are shown by closed and open bars, respectively. Insert A: SDS−PAGE analysis of cell extracts of 1 h pulse-labeled cells. Inserts B and C: SDS−PAGE analyses of culture medium from 8 h chased cells in the absence or presence of β-mercaptoethanol, respectively. Lanes: 1, 10 μg/mL vitamin K; 2, no addition; 3, 0.5 μM warfarin; 4, 1 μM warfarin; 5, 5 μM warfarin; 6, 10 μM warfarin.

intracellular protein C synthesized under warfarin treatment was 5 kDa smaller than that under vitamin K treatment. In both cases, intracellular protein C was detected as the single-chain form, but in the 8 h chased medium, we noted a two-chain form in addition to the single-chain form of recombinant protein C. Furthermore, two types of heavy chains with apparent molecular masses of 41 and 37 kDa (due to heterogeneity of sugar chains) were observed, as described (Miletich & Broze, 1990; McClure et al., 1992). When the warfarin concentration was increased, the 23 kDa light chain converted to a 20.5 kDa form (Figure 2, insert C). Taking the total amount of radioactivity of 8 h chased control cells without the addition of vitamin K or warfarin as 100%, we determined the relative radioactivities of vitamin K-treated and warfarin-treated cells, as shown in Figure 2. In the presence of vitamin K, the quantity of secreted protein C increased 1.8-fold compared to the value in control cells. In contrast, a decrease in the total amount of radioactivity was observed concomitant with an increase in warfarin concentration. At a concentration of 10 μM warfarin, 72% of the radioactivity disappeared during an 8 h chase, compared to that in control cells.

To examine the warfarin-induced degradation, further pulse−chase experiments were done in the presence of 10 μg/mL vitamin K or 1 μM warfarin. A selected clone of BHK cells, which produced a high level of protein C, was used for the pulse−chase experiments (Figure 3). The amount of radioactivity in pulse-labeled intracellular protein bands, in the presence of either vitamin K or warfarin, was almost equal and gave a ratio of vitamin K-treated to warfarin-treated cells of 1:1.15 ± 0.16 (n = 4, data not shown). On SDS−PAGE, all of the pulse-labeled protein C in vitamin K-treated cells was secreted into the medium (Figure 3A,C), while in the presence of warfarin, only part of the pulse-labeled protein C was detected in the medium (Figure 3B,D). A quantitative analysis of pulse−chase data showed that the majority of the pulse labeled radioactivity was recovered from the medium after a 4 h chase, and the total amount of radioactivity was maintained during a 24 h chase of the vitamin K-treated cells (Figure 3E). In the presence of warfarin, intracellular protein C bands disappeared at a similar rate as those of vitamin K-treated cells. The time for 50% disappearance of the radioactivity in cell extracts was estimated to be 2.5 and 2.7 h for vitamin K-treated and warfarin-treated cells, respectively. However, little protein C was secreted into the medium of warfarin-treated cells, and a decrease in the total amount of radioactivity was observed (Figure 3F). The time for 50% disappearance of the total radioactivity in warfarin-treated BHK cells was estimated to be 5.7 h. In the case of pooled stable 293 cells, essentially the same phenomena were observed, and the time for 50% disappearance of the total radioactivity in warfarin-treated cells was estimated to be 13.1 h (data not shown). However, in warfarin-treated 293 cells, the radioactivity of cell extracts disappeared much more slowly than was seen in the vitamin K-treated cells; the time for 50% disappearance of the radioactivity in cell extracts was estimated to be 1.7 and 10.0 h for vitamin K-treated and warfarin-treated cells, respectively (data not shown). These results suggest that protein C synthesized in the presence of warfarin was selectively degraded in an intracellular compartment(s), both in human and in hamster kidney cells.

Effects on the Secretion and Degradation of Protein C of Various Inhibitors for Intracellular Transport, Lysosomal Enzymes, Redox State, and Proteases. To localize the site and to identify the protease(s) responsible for the warfarin-induced intracellular degradation of protein C, we examined the effect of various inhibitory reagents on the degradation of protein C, using stable BHK cells (Figure 4A, B). By taking the amount of radioactivity in cell extracts derived from 1 h pulse-labeled cells as 100%, 8.9 ± 2.2% and 86 ± 22% (n = 7) of the radioactivity were detected in cell extracts and in medium, respectively, of 8 h chased vitamin K-treated cells. In contrast, in the presence of warfarin, 8.6 ± 1.8% and 10.7 ± 6.1% (n = 7) of the radioactivity were detected in the 8 h chased cell extracts and medium, respectively. Therefore, in the warfarin-treated cells there was an 81% disappearance of the pulse-labeled radioactivity in the 8 h chase period. In the presence of brefeldin A, an inhibitor of intracellular transport by the redistribution of Golgi proteins into the ER and prevention of further movement through the secretory pathway (Misumi et al., 1986), protein C accumulated in the vitamin K-treated cells, and 54% and 62% of the radioactivity were recovered from the cell extracts of an 8 h chase in the presence of 2 and 10 μg/mL brefeldin A, respectively. In contrast, in warfarin-treated cells, 74% and 84% of the total radioactivity disappeared during an 8 h chase in the presence of 2 and 10 μg/mL brefeldin A,

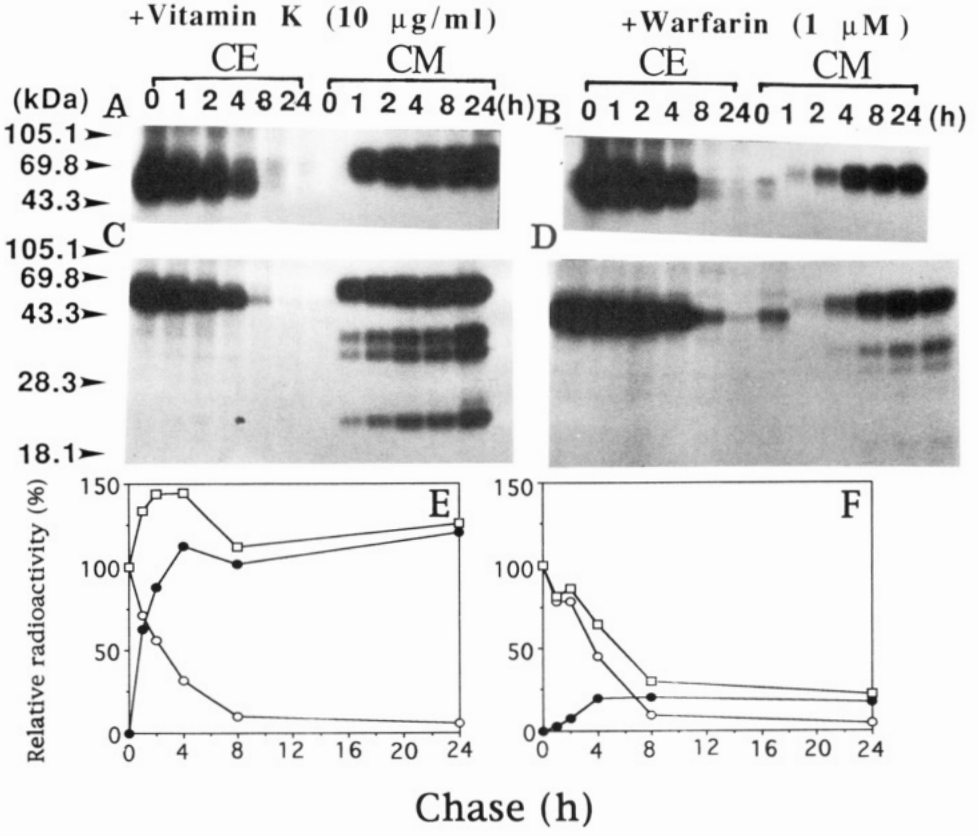

FIGURE 3: Pulse−chase and kinetic analyzes of protein C secretion in the presence of 10 μg/mL vitamin K or 1 μM warfarin. Stable BHK cells were pulse-labeled for 1 h and chased for 0, 1, 2, 4, 8, and 24 h in the presence of 10 μg/mL vitamin K (A, C, and E) or 1 μM warfarin (B, D, and F). The labeled protein C in cell extracts (CE) and conditioned medium (CM) was immunoprecipitated, as described in Materials and Methods, and analyzed on SDS−PAGE in the absence (A and B) or presence (C and D) of β-mercaptoethanol. In kinetic analyses (E and F), the amount of radioactivity in the pulse-labeled cell extracts was taken as 100%, and relative radioactivities of intracellular and secreted fractions are shown by open and closed circles, respectively. The sum of the radioactivities of both fractions at each time is shown by open squares.

respectively, thereby suggesting that protein C synthesized in the presence of warfarin was selectively degraded and that brefeldin A had no apparent effect on the degradation (Figure 4A).

Lysosomotropic agents, chloroquine (50 and 100 μM), and NH_4Cl (10 and 30 mM), which accumulate in lysosomes and elevate the intravesicular pH by virtue of their weak base properties and then reduce lysosomal protease activities (Seglen et al., 1979), showed no inhibitory effect on the secretion of vitamin K-supplemented BHK cells nor on the warfarin-induced degradation of protein C (Figure 4A). Moreover, inhibitors of endosomal and lysosomal proteases, chymostatin (50 μM) and leupeptin (50 μM), also showed no effect on protein C degradation (Figure 4B). These results are inconsistent with a lysosomal or an autophagic degradation of protein C, and the contention that degradation occurred at the pre-Golgi site is given support.

The lumen of the ER in a reducing environment is maintained by glutathione. Diamide causes rapid, stoichiometric, oxygen-independent oxidation of glutathione to an oxidized form, and diethyl maleate reacts with glutathione irreversibly and depletes it from the cells (Kosower & Kosower, 1969; Makino et al., 1994). As treatment with diamide or diethyl maleate inhibited protein C degradation and led to an accumulation of intracellular radioactivity in a dose-dependent manner, the reducing milieu of ER seems to be necessary for the degradation process of protein C. These agents, however, did not stimulate the secretion of warfarin-treated cells. 3-Amino-1,2,4-triazole (5 mM), which irreversibly inhibits catalase, another antioxidant enzyme in the peroxisome (Schonbaum & Chance, 1976), showed no effects.

To determine the specificity of degradation, various types of membrane-permeable protease inhibitors were examined (Figure 4B). ALLM, ALLN, DCI, and TPCK were dose dependently effective on warfarin-induced protein C degradation. While DCI and TPCK had an inhibitory effect on the secretion of protein C in vitamin K-supplemented cells, ALLM and ALLN inhibited the degradation, with no disturbance in the secretion from vitamin K-treated cells. In the presence of 250 μM ALLM or ALLN, almost all of the radioactivity of warfarin-treated cells was maintained without degradation even after an 8 h chase, but the amount of the secreted fraction did not increase in the presence of ALLM or ALLN. Thus, these inhibitors prevented intracellular degradation, and intracellular protein C accumulated. Other protease inhibitors against aminopeptidase (amastatin), cysteine proteases (antipain and E-64), elastase (elastatinal), and metal proteases (phosphoramidon) had no effect on the warfarin-induced degradation of protein C or on secretion from vitamin K-treated BHK cells (Figure 4B).

Subcellular Localization of Protein C. To localize the distribution of protein C in cells, endoglycosidase H digestion of intracellular and secreted protein C synthesized was done in the presence of 10 μg/mL vitamin K or 1 μM warfarin (Figure 5). In both cases, intracellular protein C bands on

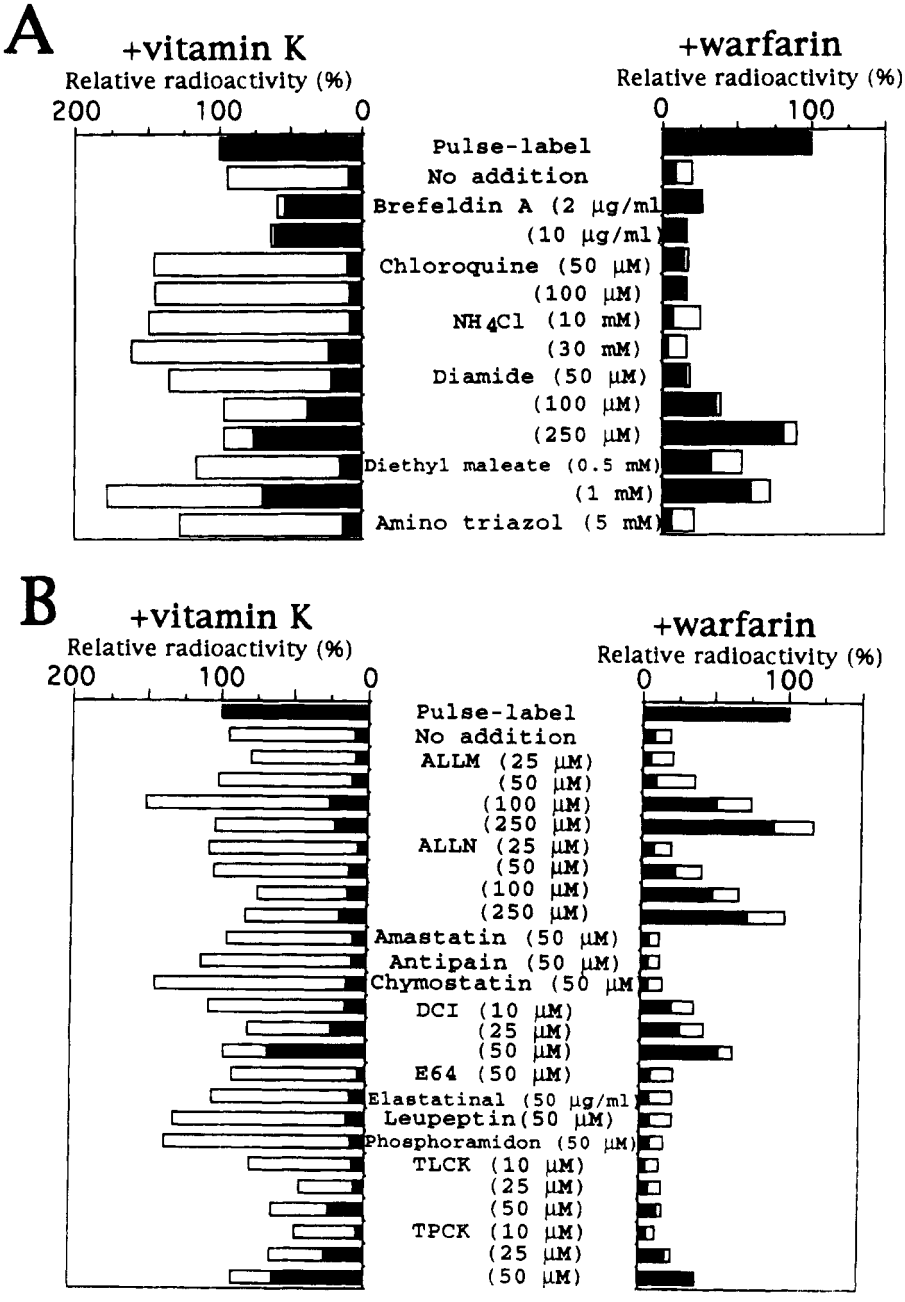

FIGURE 4: Effects of inhibitors for intracellular transport, lysosomal enzymes, and redox state (A) or for protease (B) on the secretion and degradation of protein C. Stable BHK cells (2.5×10^5) were pulse-labeled for 1 h and chased for 8 h in the presence of 10 μg/mL vitamin K or 1 μM warfarin with various inhibitors. Brefeldin A, ALLM, ALLN, DCI, amastatin, and chymostatin were dissolved in dimethyl sulfoxide. Diethyl maleate, TLCK, and TPCK were dissolved in ethanol, and others were aqueous solutions. Final concentrations of dimethyl sulfoxide and ethanol were less than 0.25% (v/v), and these solvents had no untoward effect under the conditions used. NH_4Cl and chloroquine were present in both pulse and chase periods, and others were present only in the chase period. The relative radioactivities from intracellular and secreted fractions are shown by closed and open bars, respectively. The amount of pulse-labeled radioactivity was taken as 100%.

SDS−PAGE shifted to a lower molecular mass (by 10 kDa) upon endoglycosidase H digestion (Figure 5, lanes 2 and 4). However, secreted protein C bands were resistant to the enzyme (Figure 5, lanes 5−8). These data suggest that intracellular protein C had carbohydrate chains of the high mannose type and were localized in the ER to the *cis*-Golgi compartment.

To further localize the intracellular form of the protein C, we performed immunofluorescence staining using transiently transfected 293 cells cultured in the presence of 1 μM warfarin (Figure 6). Protein C synthesized in the presence of warfarin was distributed in the reticular organelle throughout the cytoplasm but not in the nucleus (Figure 6A,C). These patterns were identical with that of PDI, the ER-resident protein (Figure 6B), but not with that of FITC−wheat germ agglutinin, the Golgi marker, which reacts with a complex type carbohydrate (Figure 6D). In the presence of vitamin K, there was no difference in subcellular localization from that of warfarin-treated 293 cells (data not shown). These results also suggest that, in either case, recombinant protein C precursor was predominantly localized in the ER.

DISCUSSION

Warfarin is a widely prescribed antithrombotic agent. The response to warfarin treatment differs among vitamin K-dependent proteins, i.e., the plasma levels of factor VII and

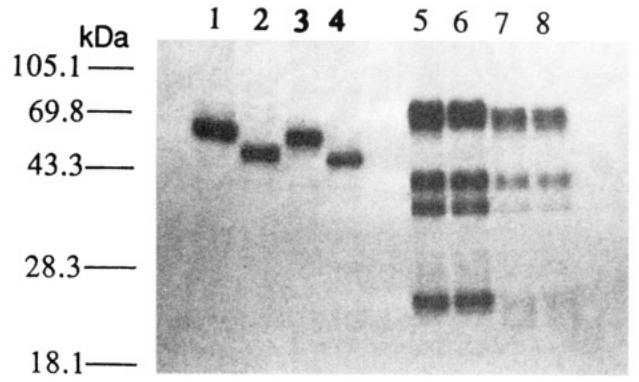

FIGURE 5: Endoglycosidase H digestion of protein C synthesized in the presence of warfarin. Stable BHK cells (2.5×10^5) were pulsed-labeled for 1 h and chased for 8 h in the presence of 10 μg/mL vitamin K (lanes 1, 2, 5, and 6) or 1 μM warfarin (lanes 3, 4, 7, and 8). Pulse-labeled cell extracts (lanes 1−4) and 8 h chased conditioned medium (lanes 5−8) were immunoprecipitated with anti-human protein C IgG and subjected to endoglycosidase H digestion. Odd and even lanes show SDS−PAGE of samples before and after endoglycosidase H digestion, respectively.

proteins C and Z drop more quickly upon warfarin administration than do those of other proteins (Viganò et al., 1984; Miletich & Bronze, 1987). The effects of warfarin on the expression and secretion of vitamin K-dependent factors have been examined. Fair and Bahnak (1984) reported that vitamin K and warfarin treatments affect prothrombin transcription in human hepatoma HepG2 cells, while Jamison et al. (1992) suggested that vitamin K and warfarin affect the rate of prothrombin synthesis, coupled with potential effects on translation. Miletich and Broze (1990) showed that warfarin treatment of HepG2 cells resulted in a dramatic decrease in secreted protein C, which did not appear to be an intracellular degradation. Grinnell et al. (1990) extensively characterized the expression and secretion of recombinant protein C in 293 cells, and they showed that a vitamin K-dependent step is required for efficient secretion and that warfarin treatment results in a 75% decrease in recombinant protein C secretion. All of these findings taken together demonstrate the inhibitory effect of warfarin on secretion, but the molecular mechanisms involved in the warfarin-induced decrease in vitamin K-dependent factors are not well understood.

We found that warfarin causes a decrease in secreted protein C in transient and in stable human and hamster kidney cells (Table 1 and Figure 2), and the addition of vitamin K, an active cofactor for vitamin K-dependent carboxylase, enhances the secreted protein C by as much as 1.8-fold, as reported by McClure et al. (1992). This value is consistent with the finding that vitamin K treatment of HepG2 cells led to a 2-fold increase in secreted prothrombin (Fair & Bahnak, 1984). In transient 293 cells, warfarin treatment caused a 2.3-fold accumulation of intracellular protein C (Table 1). It has been reported that, in the livers of warfarin-administered rats, factor X precursor increased to 265% in microsomes and decreased to 40% in plasma, but prothrombin accumulated to only 125−140% in microsomes (Stanton & Wallin, 1992). The warfarin-induced intracellular accumulation is likely to be influenced by factors such as cell species, transfection methods, and warfarin concentration and it is likely to be variable among vitamin K-dependent proteins. In Figures 2 and 3, the intensities of pulse-labeled protein C, in the presence of either vitamin K or warfarin, showed no difference, suggesting equal transcription and translation rates. The 20.5 kDa light chain observed concomitant with the increase in warfarin concentration may reflect the under-γ-carboxylated form (Figures 2 and 5), and a part of such a protein C seemed to be secreted into the medium. This observation parallels the findings that after warfarin therapy protein C decreased to 53% of normal and 54% of the total remaining antigen adsorbed to barium citrate, suggesting that a part of the under-γ-carboxylated protein C is secreted into plasma (Miletich & Broze, 1987).

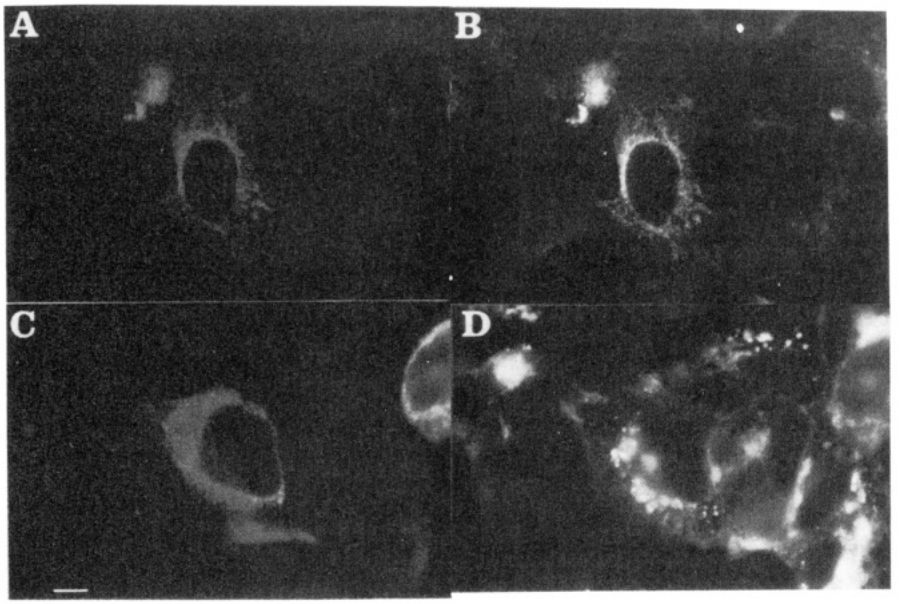

FIGURE 6: Immunofluorescent localization of protein C synthesized in the presence of warfarin. Human kidney 293 cells were transiently transfected with pcD2-PC. Two days after transfection, cells were fixed, permeabilized, and subjected to immunofluorescence staining. Panels A and B were stained with goat anti-human protein C serum and with rabbit anti-bovine PDI serum, respectively, each followed by FITC-labeled donkey anti-goat IgG and rhodamine-labeled sheep anti-rabbit IgG, respectively. Panels C and D were first stained with rabbit anti-human protein C IgG, followed by rhodamine-labeled sheep anti-rabbit IgG (panel C) and FITC-labeled wheat germ lectin (panel D). Panels A and C show protein C immunofluorescence. Panels B and D show the staining patterns of PDI and FITC−wheat germ lectin, respectively. Scale bar in panel C: 10 μm.

Figures 2−4 indicate that protein C precursor synthesized in the presence of warfarin is degraded selectively, that the degradation process is in the nonlysosomal, pre-Golgi compartment, and that a cysteine protease(s) is responsible for the degradation. Furthermore, Figures 5 and 6 suggest that, in warfarin-treated cells, protein C precursor is predominantly localized in the ER. All of these data indicate that the majority of human protein C precursor synthesized in the presence of warfarin is degraded in the ER. Quite recently, Zhang and Suttie (1994) also reported the same phenomenon in the course of rat prothrombin secretion from rat hepatoma (H-35) cells.

ER-associated protein degradation is well characterized for several proteins, e.g., T-cell antigen receptor (Klausner & Sitia, 1990), the Z variant of α_1-protease inhibitor (McCracken et al., 1989), unassembled asialogycoprotein receptor (Amara et al., 1989), 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Inoue et al., 1991), some lysozyme mutants (Omura et al., 1992), apolipoprotein B (Sakata et al., 1993), and IgM (Amitay et al., 1992; Gardner et al., 1993). This degradation of newly synthesized proteins in the ER is referred to as "quality control" and is distinct from lysosomal degradation. Some proteases are suggested to be responsible for the degradation. A cysteine protease(s) that is inhibited by ALLM and ALLN has been implicated in the degradation of HMG-CoA reductase (Inoue et al., 1991), apolipoprotein B (Sakata et al., 1993), and IgM (Amitay et al., 1992), and a serine protease(s) that is inhibited by DCI, TPCK, and TLCK is a candidate for IgM light chain breakdown (Gardner et al., 1993). ER-60 (Urade & Kito, 1992) and ER-72 proteases (Urade et al., 1993), which degrade the ER resident proteins such as PDI and calreticulin, are inhibited by typical cysteine protease inhibitors like ALLN, E64, leupeptin, iodoacetic acid, and (*p*-chloromercuri)benzoate and are suggested to be good candidates for the degradation of HMG-CoA reductase, apolipoprotein B, and IgM. In our experiments, ALLM and ALLN specifically inhibited the warfarin-induced degradation of protein C without disturbing the secretion of normal protein C from vitamin K-treated BHK cells. Therefore, it is likely that the same cysteine protease(s) as that for HMG-CoA reductase, apolipoprotein B, and IgM may degrade under-γ-carboxylated protein C molecules. We conclude that abnormal protein C precursor synthesized in the presence of warfarin is selectively degraded by a cysteine protease(s) in the ER through a "quality control" mechanism. Further studies were underway to search for a protease(s) responsible for the degradation.

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