J. Biochem. **140**, 421–427 (2006)
doi:10.1093/jb/mvj175

Features of a Newly Cloned Pig C1 Esterase Inhibitor

Chizuko Kobayashi[1], Katsuyoshi Matsunami[1], Takeshi Omori[2], Shino Nakatsu[1], Kengo Nakahata[1], Hengjie Xu[1], Ryota Shirakura[1], Masahiro Fukuzawa[2] and Shuji Miyagawa[1,*]

[1]Division of Organ Transplantation, Department of Molecular Therapeutics, and [2]Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871

Received July 19, 2006; accepted July 20, 2006

The pig cDNA encoding C1 esterase inhibitor (C1-INH) was isolated and the homology of the sequence was compared with that from other animals. The structure of pig C1-INH contains a two disulfide bridge pattern identical to the human C1-INH. In the amino acid sequence of the first Cys-91 to the C-terminal end, the pigC1-INH has a 76.2% homology with the human protein, and the sequence of the reactive site is close to the human. A surface-bound form of pig and human C1-INH, pC1-INH-PI and hC1-INH, respectively, were next constructed. Stable Chinese hamster ovarian tumor (CHO) cell lines and pig endothelial cell (PEC) lines expressing these C1-INH-PI were prepared by transfection. The basic function and the species specificity of pCI-INH were then investigated using these transfectants. pC1-INH and hC1-INH have almost the same suppressive effect on pig, human, dog and rabbit sera in complement-dependent cell lysis, indicating little species specificity.

Key words: blood coagulation, complement, gene expression, new effects of biomaterials, structure of glycoproteins.

Abbreviations: CRP, complement regulatory protein; PI, glycosylphosphatidylinositol; C1-INH-PI, PI-anchored C1 esterase inhibitor; pC1-INH, pig C1-INH; hC1-INH, human C1-INH; DAF, decay accelerating factor; CHO, Chinese hamster ovarian tumor; PEC, pig endothelial cell; AA, amino acid; LDH, lactate dehydrogenase.

In a previous study, the efficacy of the cell surface–bound form of plasma human complement regulatory proteins (CRPs) was demonstrated (*1*), such as the phosphatidylinositol (PI)-anchored C4b binding protein (C4bp-PI) (*2*), in xenotransplantation studies. As a result of the encouraging results, other PI-anchored plasma CRPs, factor H (factor H-PI), factor I (factor I-PI) and the C1 esterase inhibitor (C1-INH-PI), were also examined (*3–5*). Among these CRPs, the use of C1-INH-PI in avoiding xenograft rejection may offer several advantages. C1-INH is not only an inhibitor of the classical complement cascade, but is also a regulator of the coagulation system, which is also a contributor to hyper acute rejection (HAR) (*6*). That is, C1-INH inhibits factors XIIa, XIa and plasmin, in a manner similar to other serine protease inhibitors of the antithrombin III–α1-antitrypsin family (*7*). Therefore, the efficient down-regulation of HAR occurs when xenogenic cells express C1-INH-PI in a robust manner, and this might resolve several factors associated with acute vascular rejection (AVR) (*8*).

On the other hand, studies of pig CRP became necessary. The use of an a bioartificial liver (*9–13*) with pig liver cells in the treatment of fulminant hepatic failure will require research on plasma CRPs of the pig, because the liver produces the majority of the complement components and plasma CRPs. In addition, in the near future, the pig might be used as a scaffold for the regeneration of human tissue and organs.

In this study, pig C1-INH (pC1-INH) was cloned and some relevant features of the molecule were characterized, especially its cross-species regulation, in comparison with a human C1-INH (hC1-INH) and a membrane bound form of CRP, decay accelerating factor (DAF:CD55).

MATERIALS AND METHODS

Cell Culture—Chinese hamster ovary (CHO) cells obtained from the American Type Culture Collection (Bethesda, MD, USA) and a pig endothelial cell (PEC) line, MYP30 (*14*), were cultured in Ham's F12 medium or DMEM, respectively, containing 10% fetal bovine serum (FBS) with L-glutamine and kanamycin/amphotericin. Cultures were maintained in a 5% CO_2/95% air atmosphere at 37°C.

Cloning of C1-INH cDNA—The amino acid (AA) sequence of pCI-INH was queried in dbEST (NCBI) using the TBLASTIN program to search for cDNA fragments of pC1-INH. Based on the EST cloning data, primers for pC1-INH were identified. Total RNA was then isolated from a fresh pig liver using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). An oligoTex-dT30 mRNA purification kit was used for the mRNA preparation, following the manufacture's recommended protocol (Takara, Tokyo, Japan). A portion of purified liver mRNA was then reverse-transcribed using RevertraAce reverse-transcriptase (TOYOBO, Osaka, Japan) and a random oligo primer. A PCR experiment was performed to isolate the candidate cDNA clones using pyrobest DNA polymerase (TOYOBO,

*To whom correspondence should be addressed. Tel:+81-6-6879-3062, Fax: +81-6-6879-3069, E-mail: miyagawa@orgtrp.med.osaka-u.ac.jp

Osaka, Japan) with the following primers for pig C1-INH: (sense primer) pC1-INH5-1: 5′-CAGGTCCGGGCTGACGTC-3′ and pC1-INH5-2: 5′-GCTGACGTCGCCGCCCAG-3′, (antisense primer) pC1-INH3-1: 5′-AAGCAGGTCCCCTCACCAGAC-3′ and pC1INH3-2: 5′-CTGCTGTATTTGTACCAGAGGAAG-3′ (*15*).

The amplified DNA fragments were subcloned into the *Eco*RV site of the pBluescript II SK(–) cloning vector by a TA-cloning method. The nucleotide sequences were determined by the dideoxy chain termination method using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Construction of pC1-INH-PI—We established cDNA of C1-INH-PI with a FLAG-tag epitope, consisting of the eight-AA sequence, DYKDDDDK. This marker was designed so as to achieve maximum hydrophilicity (*16*).

In a typical run, pCXN2L (mammalian expression vector, chicken beta actin promoter and CMV enhancer with the neomycin resistant gene) (*17*)/FLAG-PI was initially constructed, as follows. The cDNA of DAF was prepared, subcloned into pCXN2L, and the short consensus repeat (SCR) 1–4 region after the signal peptide of DAF was switched to the FLAG code with an *Xho*I site. The reaction products of delta-1-90AA of pC1-INH and delta-1-99AA of hC1-INH were next flanked by the *Xho*I site and cloned into the pCXN2L/FLAG-PI. The constructed DNA sequences were confirmed by means of an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). These plasmids were transformed into *Escherichia coli* C600 and amplified using standard techniques (*5*).

Transfection of CHO Cell and PEC—The resulting plasmids were introduced into CHO cells and PEC by lipid-mediated DNA transfection with lipofectamine (LIPOFECTAMINE™ Reagent, Invitrogen, Carlsbad, CA, USA). The transfected cells were maintained in complete medium for several days in an atmosphere of humidified 5% CO_2 at 37%. The cells were then transferred to a complete medium containing 1.0 mg/ml G418 (Nacalai Tesque, Kyoto, Japan) for selection. The expression of C1-INH was confirmed by flow cytometry as described below.

Flow Cytometry—Transfected cells (1×10^6) were incubated with 1 μg of the anti-FLAG monoclonal antibody (mAb), m2, or anti-DAF mAb, 1C6 (Wako, Tokyo, Japan), for 45 min at 4°C and subsequently stained with 1.25 μg of an FITC-labeled second antibody for 45 min at 4°C. The resulting stained cells were analyzed using a FACS Calibure flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

Immunoblotting—The protein content of the transfectant and naive cell lysates were quantified by the BCA method (Pierce, Rockford, IL, USA) and 75 μg aliquots of the obtained proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a gel concentration of 10% under reducing conditions for C1-INH-PI. The separated proteins were then electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membrane was blocked in 5% skim milk in Tris-buffered saline/0.05% tween 20 (TBST) for 1 h at 25°C and then incubated in 1% bovine serum albumin (BSA)/0.5% skim milk/TBST with the anti-FLAG mAb, for 1 h at 25°C. After washing, the blots were incubated with a horseradish peroxidase conjugated secondary antibody and the signal was developed using an ECL detection system (Amersham, Princeton, NJ, USA) (*5*).

Lactate Dehydrogenase (LDH) Assay—This assay was performed following a previously described method, using a MTX-LDH kit (Kyokuto, Tokyo, Japan). The transfected cells were plated at a concentration of 2×10^4 per well in a 96-well tray, 1 day prior to the assay. After 15 h, the plates were incubated with 10% human, pig, mouse, dog and rabbit sera diluted in serum free medium with or without anti–CHO cell antiserum at individual dilutions, for 2 h at 37°C, and the released LDH was then determined. The spontaneous release of LDH activity from the target cells was less than 5% of the maximal release of LDH activity, determined from a complete lysis by sonication. Antiserum against the CHO cells was prepared from a rabbit that had been immunized with CHO cells (*4*, *5*).

Statistics—Data are presented as the mean ± SEM. The Student-*t* test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Isolation of Pig C1-INH cDNA—Several expressed sequence tag (EST) sequences containing the 5′ or 3′-terminal region with similarity to human C1-INH were identified from the DNA database by BLAST search. Oligo DNA primers were then designed, and the pC1-INH sequence was amplified from a pig liver cDNA. The cDNA fragments obtained were subcloned into the plasmid vector and the sequence was analyzed by testing the multiple independent clones.

From the nucleotide sequences, the predicted open reading frame for pC1-INH consisted of 492 AA (Fig. 1).

Alignment of the AA sequences from pig was next compared with those of human, mouse, rabbit C1-INH and bovine factor XIIa inhibitor (Fig. 2). While the overall identity of the deduced AA sequence of pC1-INH with the human C1-INH, mouse C1-INH and bovine factor XIIa is 56.9%, 58.7% and 56.3%, respectively, the homology of the AA sequence from the first Cys-91 to the C-terminal end of pigC1-INH is 76.2%, 70.4% and 67.7% with human, mouse and bovine, respectively.

Cell Surface Expression of pC1-INH-PI and hC1-INH-PI Molecules—The cDNAs of pC1-INH-PI and hC1-INH-PI are composed of delta-1-90 AA of pC1-INH or delta 1-99 AA of hC1-INH, respectively, with the FLAG tag sequence in the N-terminal and the PI-anchor of DAF in the C-terminal. The expression plasmid containing pC1-INH-PI or hC1-INH-PI (Fig. 3) was transfected into CHO cells and PECs. Stable CHO transfectants with pC1-INH-PI or hC1-INH-PI, and PEC transfectants with pC1-INH-PI were established, and reacted with the anti-FLAG mAb. Typical flow cytometric histograms for these transfectant cells are shown (Fig. 4A).

Immunoblotting Analysis—An immunoblotting analysis was performed to check the size of both constructs, hC1-INH-PI and pC1-INH-PI, using cell lysates from delegate transfectants, CHO with hC1-INH-PI#1 and pC1-INH-PI#1. Immunoblots showed major bands that were consistent with the expected molecular weight. No significant difference in molecular size was observed between the

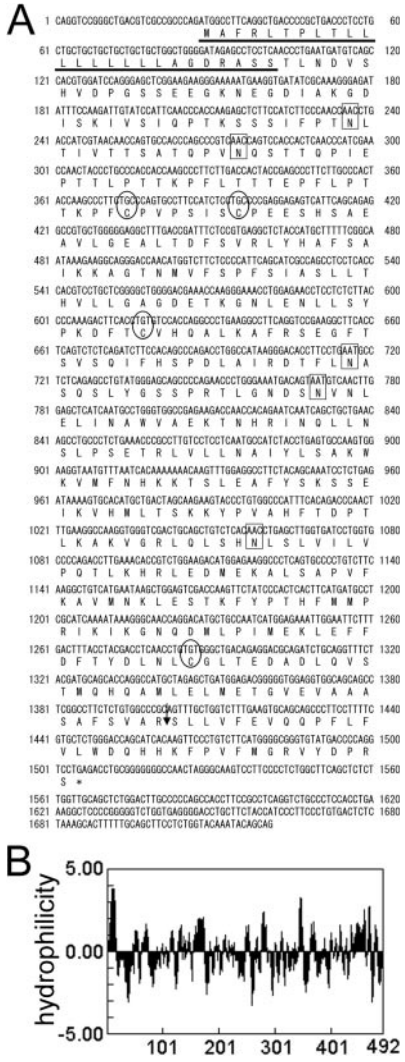

Fig. 1. **DNA and deduced amino acid sequences, and hydropathy plots of pig C1-INH.** (A) The cloned DNA and amino acid sequences of pC1-INH are shown. The signal peptide is underlined. Cysteins residues are encircled. The predicted *N*-glycosylation sites found by the NetNGlyc program are enclosed in a square, and the catalytic domain is also marked with an arrowhead (↓). (B) Kyte-Doolittle hydropathy plot of pC1-INH.

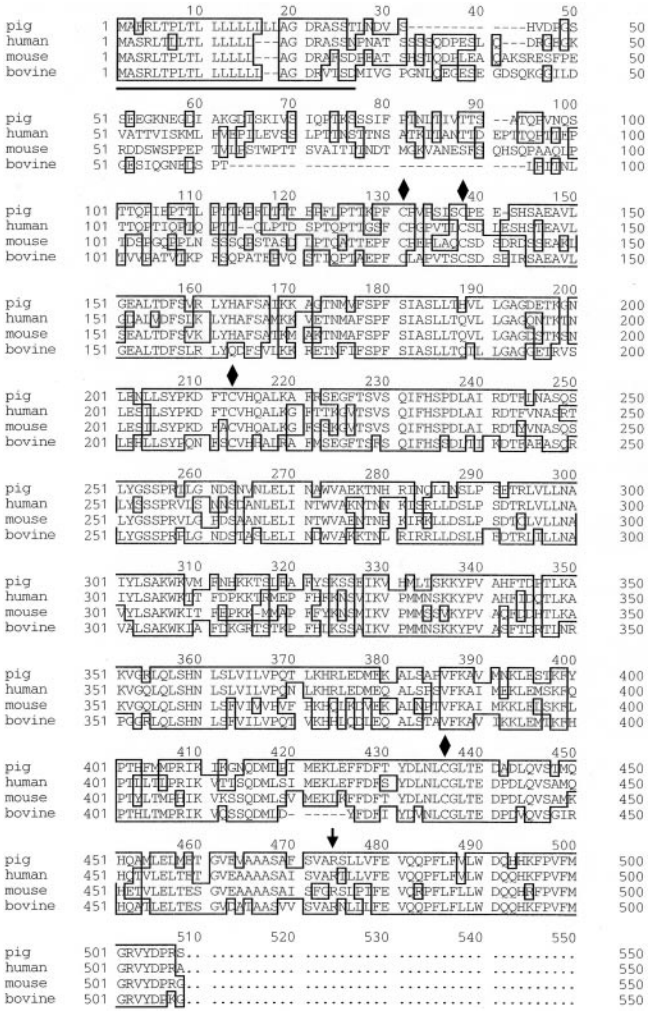

Fig. 2. **Alignment of the AA sequences from pig, human, mouse, rabbit C1-INH and bovine factor XIIa inhibitor.** Identical residues are boxed, the signal peptide is underlined, and cysteins residues are marked (diamonds) and the catalytic domain is also marked with an arrow (↓).

CHO with hC1-INH-PI#1 and pC1-INH-PI#1 (Fig. 4B). The blots also showed nonspecific bands.

Species Restriction of Pig and Human C1-INH—CHO cell transfectants with pC1-INH-PI and hC1-INH-PI were produced. The CHO cell transfectants were incubated with anti-CHO anti serum and each animal serum as a source of complement. Amelioration of complement-mediated lysis by the transfectant molecules was examined by an LDH assay, using pig, human, dog, mouse and rabbit sera. The pig and human C1-INH-PI molecules had a significant protective effect on the CHO membrane from complement attack by these sera. The inhibition rate of pC1#1, the high expression clone, to each serum is different, for example, 72.5 to 73.9% for pig, 54.6 to 58.2% for human, 84.1% for dog and 58.1 to 92.0% for rabbit. As a tendency, pC1#1 does not show a strong effect on human serum in comparison with the other sera. On the other hand, pC1#2 shows a relative close inhibition rate to each serum, 31.9 to 37.3% for pig, 42.6 to 51.0% for human and 36.7 to 50.6% for rabbit sera, except 21.2% for dog sera. However, on average, the inhibition rate of these two clones, which are 46.1%, 48.4%, 47.5% and 40.7%, to pig, human, dog and rabbit, imply less species selectivity.

Concerning hC1#1 and hC1#2, the inhibition rate is within a small range for all four sera, 51.6 to 77.2% and 27.3 to 36.7%, respectively. In addition, the inhibition rate of hC1#2, which shows almost the same FACS shift values as pC1#2, has a tendency to be close to those of pC1#2 in each serum.

On the other hand, DAF showed a quite strong inhibitory effect, over 90%, in pig, human, and dog sera, but only a 19.6 to 33.9% suppression to rabbit serum, suggesting that DAF has a relative clear tendency toward species specificity compared with C1-INH.

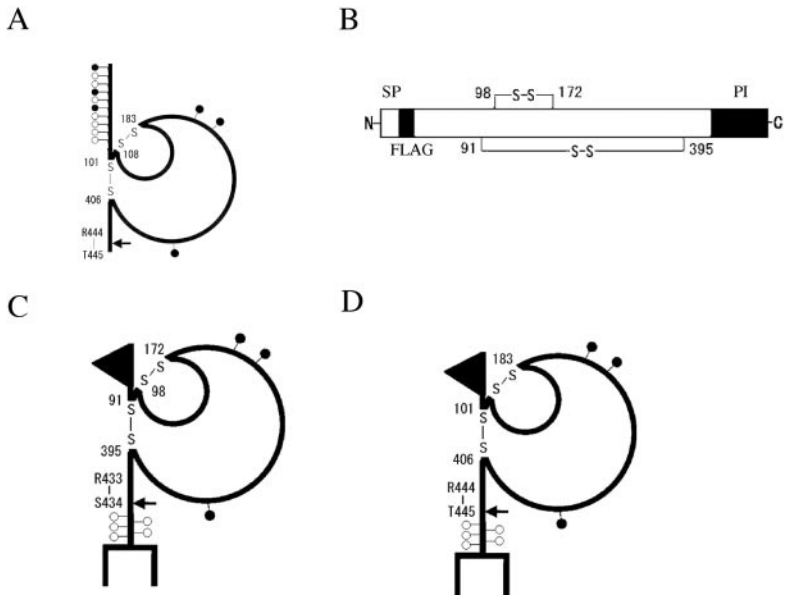

Fig. 3. **Experimental design.** (A) Schematic diagram of hC1-INH. Cysteins residues are circled. The reactive site is marked with an arrowhead (residues 444–445). *O*-Glycosylation sites are marked in white circles (open circles), and *N*-glycosylation sites are also indicated in black circles (solid circles). (B) Structure of pC1-INH-PI. The hybrid molecule contains the FLAG epitope, next to the signal peptide (SP) sequence of human DAF cDNA, a delta 1–90 amino acid sequence form of a pC1-INH cDNA and the PI-anchor region of human DAF cDNA. (C) Schematic diagram of pC1-INH-PI. (D) Schematic diagram of hC1-INH-PI. SP, signal peptide; FLAG, FLAG sequence; PI, PI-anchor region of human DAF cDNA. The disulfide bridge pattern is shown with bars. The reactive site is marked with an arrowhead. Circles denote the known sites of oligosaccharide attachment. *O*-glycosylation sites are marked in white circles (open circles), and *N*-glycosylation sites are indicated by black circles (solid circles).

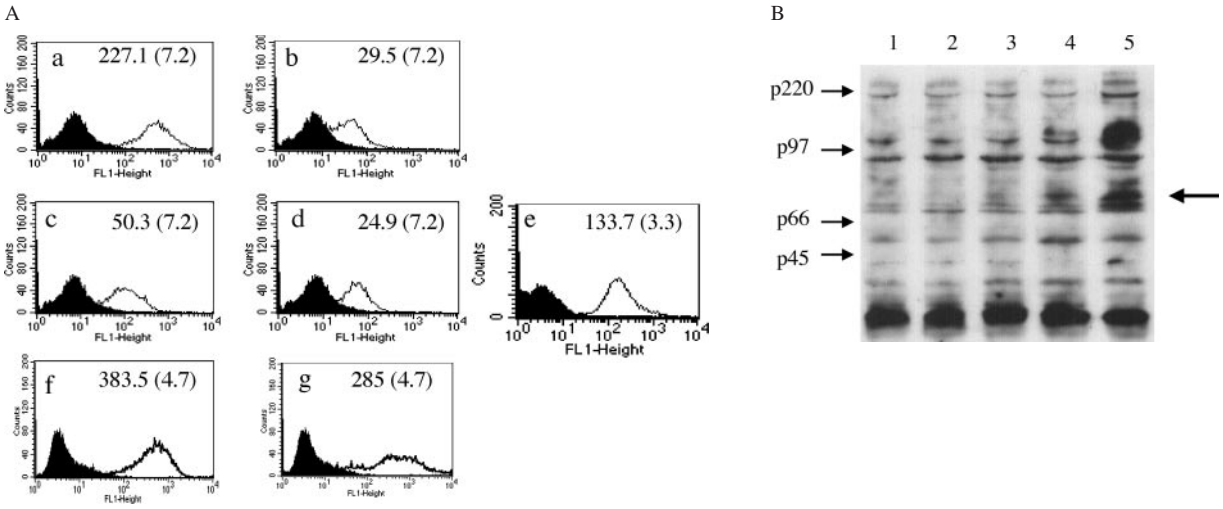

Fig. 4. **The profile of each transfectant.** (A) FACS profiles of the transfected pC1-INH-PI and hC1-INH-PI. Expression vectors containing pC1-INH-PI or hC1-INH-PI were transfected into CHO cells and PECs. The cell surface expression levels of these molecules were determined by flow cytometric analysis. Typical flow cytometric histograms for these transfectants are shown (a–g). Naive control and stable transfectants were treated with the anti-FLAG or the anti-DAF mAb, followed by a FITC conjugated rabbit anti–mouse-IgG antibody. (a) Stable CHO transfectant of pC1-INH-PI #1, (b) Stable CHO transfectant of pC1-INH-PI #2, (c) Stable CHO transfectant of hC1-INH-PI#1, (d) Stable CHO transfectant of hC1-INH-PI #2, (e) Stable CHO transfectant of DAF, (f) Stable PEC transfectants of pC1-INH-PI#1, (g) Stable PEC transfectants of pC1-INH-PI#2. (B) Western blot analysis of CHO transfectants. Naive cells and CHO cell transfectants, hC1-INH-PI #1 and pC1 INH-PI #1 were solubilized with SDS. For each lane, 40 μg of total cell lysate were loaded, and stained with an anti–FLAG-epitope monoclonal antibody. Specific bands for hC1-INPI and pC1-INH-PI were detected Lane 1, naive CHO; lane 2, CHO-DAF; lane 3, CHO-FLAG (mock); lane 4, hC1-INH-PI #1; lane 5, pC1-INH-PI #1.

On the whole, the results suggest that both the C1-INH molecules show less special species selectivity, at least on the surface of CHO cells (Fig. 5, A–D).

Trial to Express pC1-INH on PEC—The complement regulatory function of pC1-INH to human serum was examined, using PEC transfectant as an in vitro xenograft model. Amelioration of complement mediated PEC lysis by pig C1-INH-PI was investigated.

Although the CHO cell transfectant, pC1#1, does not show excellent suppression on human complement, both the PEC transfectants with pC1-INH show high expression values in FACS mean shift, and a clear suppression on PEC lysis by human complement by approximately 85%, suggesting that pig C1-INH is able to function as an efficient replacement for the corresponding human C1-INH (Fig. 6).

DISCUSSION

The homology in the AA sequence of each C1-INH in the area from the N-terminal to the first Cys-91 is very poor (*18–23*). However, in the AA sequence from the first Cys-91 to the C-terminal end, pigC1-INH has a 76.2%, 70.4% and

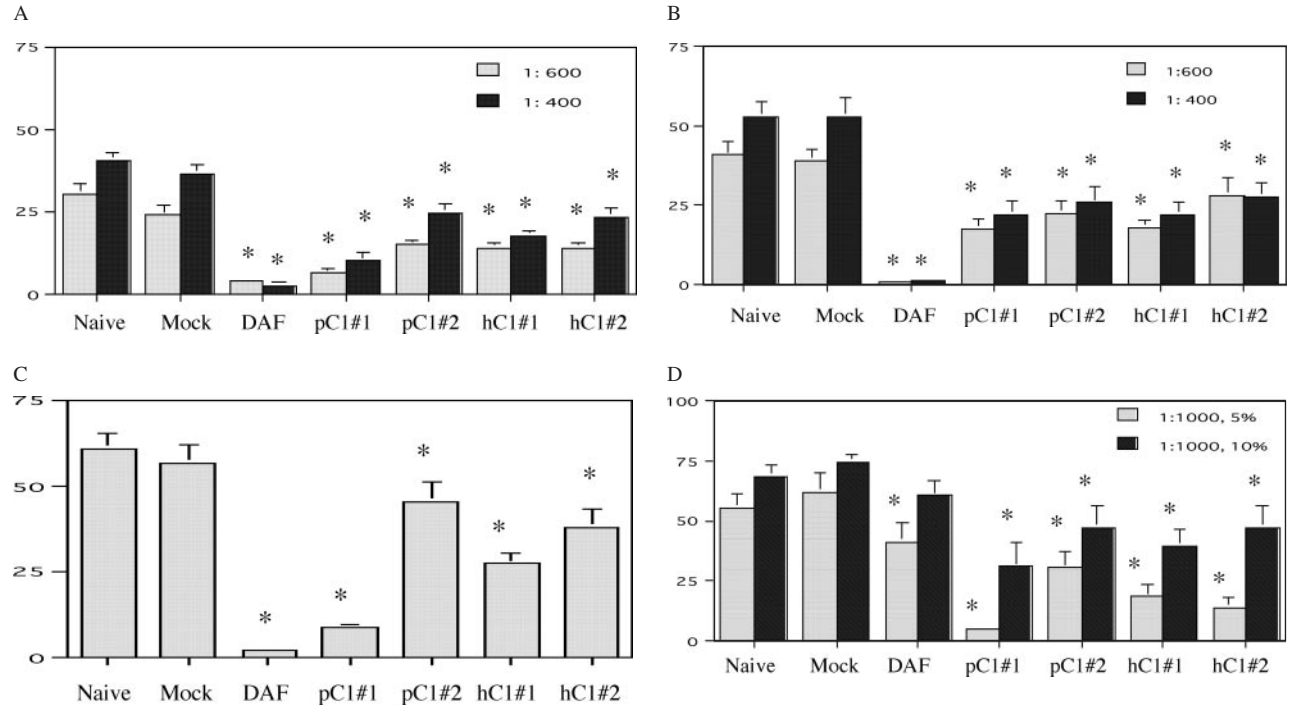

Fig. 5. **Amelioration of complement mediated CHO cell lysis by pig and human C1-INH-PI.** Naive CHO and CHO transfectants were incubated with rabbit anti-CHO serum as a source of complement activator and each serum. The % lysis was calculated by an LDH assay as described in materials and methods. pC1#1, pig C1-INH #1; pC1#2, pig C1-INH #2; hC1#1, human C1-INH #1; hC1#2, human C1-INH #2. Values are presented as the mean ± SEM. (n = 8–12). *indicates a significant differences (*vs.* naive and mock controls, $p < 0.05$). (A) Naive CHO and CHO transfectants were incubated with a 1:400 or 1:600 diluted rabbit anti-CHO serum and 10% pig serum. (B) Naive CHO and CHO cell transfectants were incubated with a 1:400 or 1:600 diluted rabbit anti-CHO serum and 10% human serum. (C) Naive CHO and CHO cell transfectants were incubated with a 1:600 diluted rabbit anti-CHO serum and 10% dog serum. (D) Naive CHO and CHO cell transfectants were incubated with a 1:1,000 diluted rabbit anti-CHO serum and 5% or 10% rabbit serum.

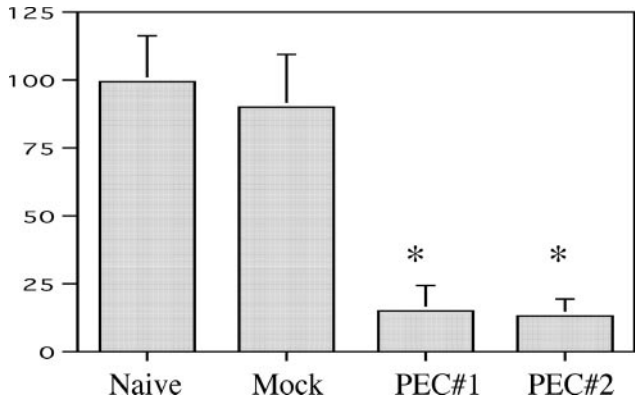

Fig. 6. **Amelioration of complement mediated PEC lysis by pig C1-INH-PI.** Naive PEC and PEC transfectants with pig C1-INH, PEC#1 and PEC#2, were incubate 10% human serum. The % lysis was calculated by an LDH assay as described in "MATERIALS AND METHODS." Values are presented as mean + SEM (n = 7). *indicates a significant differences (*vs.* naive and mock controls, $p < 0.05$).

67.7% homology with human C1-INH, mouse C1-INH and bovine factor XIIa, respectively. In addition, the structure of pC1-INH shows a disulfide bridge pattern comprised of two disulfide bridge, Cys-91 to Cys-395 and Cys-98 to Cys-172, that is identical to those of the human, mouse and bovine. However, the AA sequence at the reactive site, VARSLL, is close to the human, bovine, rabbit, but not to the mouse.

Concerning *N*- and *O*-glycosylation, the human C1-INH contains three *N*- and seven *O*-glycosylation sites before Cys-101. While pC1-INH has little similarity to the human in the N-terminal to Cys-91 region, it shows almost the same *N*-glycosylation pattern after Cys-91, suggesting that pC1-INH contains three *N*-glycosylation sites after Cys-91. The *N*-glycosylaton sites, Asn-45, 58, 205, 220 and 319, are indicated in Fig. 1. The *O*-glycosylation sites are predicted for Thr-47, 50, 51, 54, 61, 62, 68, 69, 72, 73, 78, 79, 80, 86, 87, and Ser-60 by the NetOGlyc program.

Kite-Doolittle hydropathy plots of the entire protein, shown in Fig. 1, indicate that the proteins exhibit no special topology, a common feature of fluid phase proteins.

Regarding the species-specificity of membrane CRP, DAF showed a strong complement regulatory function on not only in human serum, but in pig and dog sera as well. The degree of suppression could not be compared among these three sera in this study. On the other hand, DAF indicated only a slightly downregulation effect on rabbit serum.

From the review of other papers related to DAF, Moran *et al.* reported a strong species specificity of human DAF to mouse, rabbit and rat serum, but not guinea-pig. They could not rule out whether the observed lack of inhibition

is due to the high concentrations of serum required for hemolysis, or is an artifact of the heterologous system rather than reflecting true species-restriction. However, they verified the species-specificity of DAF(*24*).

On the contrary, an analysis of human, rat and mouse DAF by Harris *et al.* showed that human DAF inhibited the human and rat classical pathway of the complement, rat DAF inhibited rat and human classical pathway, and mouse DAF inhibited the classical pathway in all three species. Further, human DAF was the most powerful inhibitor of both human and the rat alternative pathway of complement and also inhibited the mouse alternative pathway, although less efficiently. Rat DAF inhibited only the rat alternative pathway, and mouse DAF inhibited both the rat and mouse alternative pathway. Taken together, they concluded that human and rodent DAF are not species specific in their complement-inhibiting activities (*25*). However, they also indicated that complement inhibition was best achieved by homologous DAF. In addition, homology between mouse and human DAF shared only a 50% identity at the AA level (*26*, *27*).

Moreover, Perez de la Lastra *et al.* reported a feature of pig DAF, which contains only three short consensus repeats. Pig DAF inhibited human complement, but had no significant activity against pig complement, whereas human DAF was strongly inhibitory for both human and pig complement (*28*). The function of DAF between the pig and human is somewhat complicated. However, a comparison of pig DAF with the sequence of human DAF revealed an AA identity of 64% through three short consensus repeats (*28*).

Concerning CD59, van den Berg *et al*. isolated human, rat, sheep and pig CD59, and concluded that none of the CD59 tested were species specific in their complement-inhibiting activity (*29*). However, the degree of complement inhibitory function of each CD59 on human serum was different, and the function of human CD59 on each serum also varied.

Hinchliffe *et al.* next reported data on the cloning of the cDNA for pig CD59, and concluded that human CD59 markedly inhibited lysis by human, pig and sheep complement, but only moderately inhibited lysis by rodent complement, rat, mouse and rabbit (*30*). However, human CD59 inhibits human complement more efficiently than pig complement, and the reverse is also true. In addition, the degree of conservation at the AA level between mouse CD59 and human CD59 was only 34% and between mouse CD59 and rat CD59, the value was 60% (*31*). Furthermore, the amino acid sequence of pig CD59 is 48% identical to human CD59, 46.5% identical to rat CD59, and 38% identical to murine CD59 (*30*).

Taken together, DAF and CD59 sometimes shows complement regulatory function in a cross species manner, but even under such conditions, except for a few cases, homologous DAF and CD59 achieved the best complement regulatory function, indicating the species-specificity of the membrane CRPs based on the diversity of their AA sequence.

The basic function and species specificity of pCI-INH were next investigated using CHO transfectants and several sera, because the species specificity of the CRPs in the fluid phase has not been well analyzed. In our previous study, we demonstrated that the first 100AA from the N-terminal of hC1-INH do not participate in complement regulatory function. The conformational changes of C1-INH as the result of deleting any part of the loops, namely, 108–188 and 183–406, presumably cause this molecule to become susceptible to proteases in the endoplasmic reticulum or the Golgi apparatus. In other words, the loops are strongly related to complement regulatory function, and, at the same time, serve to stabilize the conformation of this molecule (*5*). PI-anchored form of C1-INH without the first 100AA from N-terminal, pC1-INH, was then constructed.

As regards rabbit serum, it indicated a stronger complement function on CHO cells than the other sera. Therefore, a low titer of anti-CHO antibody (1:1,000) and 5% serum were used in this analysis. On the other hand, CHO cell were not lysed by mouse sera even with a high titer of anti-CHO antibody, such as 1:50 and 1:00 (data not shown). The data reported in this study indicate that the plasma CRP, C1-INH has little species-specificity at least among the pig, human, dog and rabbit, supported by the relatively high homology in the AA sequences in comparison with those of membrane CRPs. Further studies of other plasma CRPs, such as C4bp, factor H and factor I, will be needed in order to identify the general tendency of plasma CRPs in regard to species-specificity.

Finally, findings reported in previous studies suggest that the C1 step in the inhibition of the classical complement pathway is very effective and efficient, compared with the C3 step of inhibition. In addition, C1-INH can compensate for DAF in downregulating C4 fragment deposition (*5*, *6*, *31–34*). Moreover, PEC are lysed mainly by the classical pathway of human complement, because the PEC cell surface little activates the alternative pathway of human complement. On the other hand, as a feature of C1-INH-PI, the molecules depend, not on catalytic function, but reactions with target proteases to form proteolytically inactive stoichiometric 1:1 complexes as well (*35*). Thus, compared to DAF, C1-INH-PI is less efficient in the downregulation of complement-mediated cell lysis. However, our findings in this study provide that pC1-INH can function as an efficient replacement for hC1-INH.

This work was supported by Grant-in-Aids from the Ministry of Education, Science, and Culture, and Special Coordination Funds for Promoting Science and Technology and the 21st Century COE program. The authors thank Dr. Milton S. Feather for his editing of the manuscript, and Dr. Congxiao Gao and Ms. Etsuko Kitano for technical assistance.

REFERENCES

1. Miyagawa, S., Matsunami, K., Yoshitatsu, M., Mikata, S., Matsuda, H., and Shirakura, R. (2001) Attempts to prepare suitable complement regulatory molecules for clinical xenotransplantation. *J. Card. Surg.* **16**, 429–438
2. Mikata, S., Miyagawa, S., Yoshitatsu, M., Ikawa, M., Okabe, M., Matsuda, H., and Shirakura, R. (1998) Prevention of hyperacute rejection by phosphatidylinositol-anchored mini-complement receptor type 1. *Transplant. Immunol.* **6**, 107–110
3. Yoshitatsu, M., Miyagawa, S., Mikata, S., Matsunami, K., Yamada, M., Murase, A., Sawa, Y., Ohtake, S., Matsuda, H., and Shirakura, R. (1999) Function of human factor H and I on xeno-surface. *Biochem. Biophys. Res. Commun.* **265**, 556–562

4. Matsunami, K., Miyagawa, S., Yamada, M., Yoshitatsu, M., and Shirakura, (2000) R. A surface-bound form of human C1 esterase inhibitor improves xenograft rejection. *Transplantation* **69**, 749–755
5. Fukuta, D., Miyagawa, S., Yamada, M., Matsunami, K., Kurihara, T., Shirasu, A., Hattori, H., and Shirakura, R. (2003) Effect of Various forms of C1 esterase inhibitor (C1-INH) and DAF on complement mediated xenogeneic cell lysis. *Xenotransplantation* **10**, 132–141
6. Miyagawa, S., Hirose, H., Shirakura, R., Naka, Y., Nakata, S., Kawashima, Y., Seya, T., Matsumoto, M., Uenaka, A., and Kitamura, H. (1988) The mechanism of discordant xenograft rejection. *Transplantation* **46**, 825–830
7. Siddique, Z., McPhaden, A.R., and Whaley, K. (1992) A single base deletion from the C1-inhibitor gene causes type I hereditary angio-oedema. *Hum. Hered.* **42**, 231–234
8. Cooper, D.K., Gollackner, B., Knosalla, C., and Teranishi, K. (2002) Xenotransplantation—how far have we come? *Transpl. Immunol.* **9**, 251–256
9. Toyomura, K., Fujimura, T., Murakami, H., Natsume, T., Shigehisa, T., Inoue, N., Takeda, J., and Kinoshita, T. (1997) Molecular cloning of a pig homologue of membrane cofactor protein (CD46). *Int. Immunol.* **9**, 869–876
10. Chamuleau, R.A., Deurholt, T., and Hoekstra, R. (2005) Which are the right cells to be used in a bioartificial liver? *Metab. Brain. Dis.* **20**, 327–335
11. Van-de-Kerkhove, M.P., Di-Florio, E., Scuderi, V., Mancini, A., Belli, A., Bracco, A., Scala, D., Scala, S., Zeuli, L., Di-Nicuolo, G., Amoroso, P., Calise, F., and Chamuleau, R.A. (2003) Bridging a patient with acute liver failure to liver transplantation by the AMC-bioartificial liver. *Cell Transplant.* **12**, 563–568
12. Kuddus, R., Patzer, J.F., Lopez, R., Mazariegos, G.V., Meighen, B., Kramer, D.J., and Rao, A.S. (2002) Clinical and laboratory evaluation of the safety of a bioartificial liver assist device for potential transmission of porcine endogenous retrovirus. *Transplantation* **73**, 420–429
13. Khalili, T.M., Navarro, A., Ting, P., Kamohara, Y., Arkadopoulos, N., Solomon, B.A., Demetriou, A.A., and Rozga, J. (2001) Bioartificial liver treatment prolongs survival and lowers intracranial pressure in pigs with fulminant hepatic failure. *Artif. Organs.* **25**, 566–570
14. Miyagawa, S., Shirakura, R., Iwata, K., Nakata, S., Matsumiya, G., Izutani, H., Matsuda, H., Terado, A., Matsumoto, M., Nagasawa, S., and Seya, T. (1994) Effects of transfected complement regulatory proteins, MCP, DAF, and MCP/DAF hybrid, on complement-mediated swine endothelial cell lysis. *Transplantation* **58**, 834–840
15. Matsunami, K., Miyagawa, S., Nakagawa, K., Hideak, O., and Shirakura, R. (2006) Molecular cloning of pigGnT-I and I.2: An application to xenotransplantation. *Biochem. Biophys. Res. Commun.* **343**, 677–683
16. Brizzard, B.L., Chubet, R.G., and Vizard, D.L. (1994) Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *BioTechniques* **16**, 730–735
17. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199
18. Bock, S.C., Skriver, K., Nielsen, E., Thogersen, H.C., Wiman, B., Donaldson, V.H., Eddy, R.L., Marrinan, J., Radziejewska, E., Huber, R., Shows, T.B., and Magnusson, S. (1986) Human C1 inhibitor: primary structure, cDNA cloning, and chromosomal localization. *Biochemistry* **25**, 4292–4301
19. Russell, J.A., Whaley, K., and Heaphy, S. (1997) The sequence of a cDNA encoding functional murine C1-inhibitor protein. *Biochim. Biophys. Acta* **1352**, 156–160
20. Lener, M., Vinci, G., Duponchel, C., Meo, T., and Tosi, M. (1998) Molecular cloning, gene structure and expression profile of mouse C1 inhibitor. *Eur. J. Biochem.* **254**, 117–122
21. Thornton, R.D. and Kirby, E.P. (1988) Interaction of bovine factor XIIa with an inhibitor from bovine plasma. *Biochim. Biophys. Acta* **964**, 19–27
22. Thornton, R.D. and Kirby, E.P. (1987) Isolation and characterization of an inhibitor of factor XIIa from bovine plasma. *J. Biol. Chem.* **262**, 12714–12721
23. Aulak, K.S., Davis, A.E., Donaldson, V.H., and Harrison, R.A. (1993) Chymotrypsin inhibitory activity of normal C1-inhibitor and a P1 Arg to His mutant: evidence for the presence of overlapping reactive centers. *Protein Sci.* **2**, 727–732
24. Moran, P., Beasley, H., Gorrell, A., Martin, E., Gribling, P., Fuchs, H., Gillett, N., Burton, L.E., and Caras I.W. (1992) Human recombinant soluble decay accelerating factor inhibits complement activation in vitro and in vivo. *J. Immunol.* **149**, 1736–1743
25. Harris, C.L., Spiller, O.B., and Morgan, B.P. (2000) Human and rodent decay-accelerating factors (CD55) are not species restricted in their complement-inhibiting activities. *Immunology* **100**, 462–470
26. Song, W.C., Deng, C., Raszmann, K., Moore, R., Newbold, R., McLachlan, J.A., and Negishi, M. (1996) Mouse decay-accelerating factor: selective and tissue-specific induction by estrogen of the gene encoding the glycosylphosphatidylinositol-anchored form. *J. Immunol.* **157**, 4166–4172
27. Spicer, A.P., Seldin, M.F., and Gendler, S.J. (1995) Molecular cloning and chromosomal localization of the mouse decay-accelerating factor genes. Duplicated genes encode glycosylphosphatidylinositol-anchored and transmembrane forms. *J. Immunol.* **155**, 3079–3091.
28. Perez de la Lastra, J.M., Harris, C.L., Hinchliffe, S.J., Holt, D.S., Rushmere, N.K., and Morgan, B.P. (2000) Pigs express multiple forms of decay-accelerating factor (CD55), all of which contain only three short consensus repeats. *J. Immunol.* **165**, 2563–2573
29. Powell, M.B., Marchbank, K.J., Rushmere, N.K., van den Berg, C.W., and Morgan, B.P. (1997) Molecular cloning, chromosomal localization, expression, and functional characterization of the mouse analogue of human CD59. *J. Immunol.* **158**,1692–702.
30. van den Berg, C.W. and Morgan, B.P. (1994) Complement-inhibiting activities of human CD59 and analogues from rat, sheep, and pig are not homologously restricted. *J. Immunol.* **152**, 4095–4101
31. Hinchliffe, S.T., Rushmere, N.K., Hanna, S.M., and Morgan, B.P. (1998) Molecular cloning and functional characterization of the pig analogue of CD59: relevance to xenotransplantation. *J. Immunol.* **160**, 3924–3932
32. Dalmasso, A.P. and Platt, J.L. (1993) Prevention of complement-mediated activation of xenogeneic endothelial cells in an in vitro model of xenograft hyperacute rejection by C1 inhibitor. *Transplantation* **56**, 1171–1176
33. Hecker, J.M., Lorenz, R., Appiah, R., Vangerow, B., Loss, M., Kunz, R., Schmidtko, J., Mengel, M., Klempnauer, J., S. Piepenbrock, S., Dickneite, G., Neidhardt, H., Ruckoldt, H., and Winkler, M. (2002) C1-inhibitor for prophylaxis of xenograft rejection after pig to cynomolgus monkey kidney transplantation. *Transplantation* **73**, 688–694
34. Schroder, C., Pfeiffer, S., Wu, G., Zorn, G.L., Ding, L., Allen, C., Harrison, R.A., White, D.J., Azimzadeh, A.M., and Pierson, R.N. (2003) Effect of complement fragment 1 esterase inhibition on survival of human decay-accelerating factor pig lungs perfused with human blood. *J. Heart Lung Transplant.* **22**, 1365–1375
35. Harpel, P.C., Hugli, T.E., and Cooper N.R. (1975) Studies on human plasma C1 inactivator-enzyme interactions. II. Structural features of an abnormal C1 inactivator from a kindred with hereditary angioneurotic edema. *J. Clin. Invest.* **55**, 605–611