Pigs Express Multiple Forms of Decay-Accelerating Factor (CD55), All of Which Contain Only Three Short Consensus Repeats

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Pigs Express Multiple Forms of Decay-Accelerating Factor (CD55), All of Which Contain Only Three Short Consensus Repeats


We report the cloning of cDNAs encoding multiple isoforms of the pig analogue of human decay-accelerating factor (DAF; CD55). Screening of a pig muscle cDNA library using a human DAF probe identified a single clone that encoded a DAF-like molecule comprising three short consensus repeats (SCR) homologous with the amino-terminal three SCR in human DAF, a serine/threonine-rich (ST) region, and sequence compatible with a transmembrane domain and cytoplasmic tail. Northern blot and RT-PCR analysis showed that pig DAF was expressed in a wide range of tissues. Additional isoforms of DAF were sought using RT-PCR and 3′-rapid amplification of cDNA ends followed by sequencing. Isoforms containing a GPI anchor and with differing lengths of ST region were identified; no isoform containing a fourth SCR was found. Cloning of the GPI-anchored isoform from granulocytes confirmed that it was identical with the original transmembrane isoform through the three SCR and first portion of ST and was derived from a frame shift caused by splicing out 176 bp of sequence. A panel of mAbs was generated and used to analyze the distribution and anchoring of pig DAF in circulating cells. Pig DAF was expressed on all circulating cells and was transmembrane anchored on erythrocytes, but completely or partially GPI anchored on granulocytes and mononuclear cells. The transmembrane isoform of pig DAF was expressed on Chinese hamster ovary cells and was shown to affect regulatory activity for the classical pathway of human complement, but was only marginally active against pig serum.


Normal human cells and tissues express C regulatory proteins (CRP) that block the activation of C on autologous cells by inhibiting formation or promoting breakdown of the C3 convertase or by inhibiting assembly of the terminal membrane attack complex. In humans, two CRP act in tandem to inhibit the C3 convertases of the activation pathways: decay-accelerating factor (DAF; CD55) and membrane cofactor protein (MCP; CD46). These proteins, together with several other CRP and C receptors, are encoded within a gene cluster termed the regulators of C activation locus on chromosome 1q32 (2). Regulators of C activation proteins are characterized structurally by the presence of several tandem repeats of a 60-aa motif termed the short consensus repeat (SCR) (2–4). Human DAF is a GPI-linked glycoprotein of 70 kDa, comprising four SCR domains followed by a serine/threonine (ST)-rich stretch that is heavily O-glycosylated and provides a rigid structure projecting the SCR domains away from the cell membrane. DAF is widely and abundantly expressed on hemopoietic cells, endothelium, and epithelium and in diverse tissues (reviewed in Ref. 5). The C regulatory activity of DAF resides in its ability to accelerate the decay of the classical and alternative pathway C3 convertases, C4b2a and C3bBb. Several mAbs capable of inhibiting the function of DAF have been generated, and all target SCR2 or -3, indicating that this region is critical for association with the C3 convertase (6). Deletions of individual SCR also demonstrates that SCR2 and -3 are required for efficient regulation in the classical pathway, while SCR2, -3, and -4 are all required for alternative pathway regulation (7). Recent information obtained from modeling studies implicates the groove between SCR2 and -3, which contains at its base three tandem lysine residues (K125, K126, and K127) and several exposed hydrophobic residues (8).

Analogues of DAF have been isolated from erythrocytes of guinea pig (9), rabbit (10), mouse (11), and orangutan (12). Although DAF in each of these species was similar in many respects to the human protein, some interesting differences emerged. Guinea pig, rabbit, and orangutan DAF were all GPI anchored, whereas mouse DAF was not released by PIPLC in all tissues, indicating that it was in part transmembrane anchored (11). Guinea pig erythrocyte DAF was shown to comprise three distinct species with Mr of 55, 70, and 88 kDa, all of which had identical amino-terminal sequences (13). Clarification of these differences emerged from the cloning of DAF analogues from guinea pig (14), mouse (15, 16), and rat (17). In each species, several isoforms were identified, all of which contained the four-SCR structure that typifies human DAF, but with substantial variability in the ST region and the membrane anchor within and between species. In guinea pig and mouse, transmembrane and GPI-anchored isoforms were present, and in rat, guinea pig, and mouse, putative secreted forms
were found. In most species these multiple isoforms have arisen by alternative splicing in a single gene, but in the mouse there is an added complication, in that there are two DAF genes, each alternatively spliced to generate several isoforms (15, 18). An alternative splice product has also been described for human DAF, a frame shift downstream of the region encoding the SCRs and ST resulting in a hydrophilic carboxyl terminus that is predicted to encode a secreted form of the protein (19). Expression in vivo of this alternatively spliced form of human DAF has not been demonstrated.

Regulation of C in the pig has become a subject of interest because of the planned use of pig organs for transplantation to man (20). To circumvent C-mediated hyperacute rejection, an inevitable consequence of pig-human transplants, pigs are now bred that express human C regulators on endothelium (21, 22). However, the contribution of the endogenous pig inhibitors to C regulation remains to be assessed. We have undertaken characterization of membrane regulators of C in the pig and have recently described the purification and molecular analysis of the pig analogues of human MCP and CD59 (23, 24). We here set out to identify and clone the pig analogue of human DAF. Cloning from a cDNA library and sequencing from tissue mRNA identified multiple isoforms of pig DAF, including transmembrane-anchored and GPI-anchored forms. Intriguingly, pig DAF differed substantially from human DAF and all other species analogues of DAF heretofore described in that all isoforms found contained only three SCR domains. We present a comprehensive analysis of this unique form of DAF and comment on the implications of these findings to current efforts in xenotransplantation.

Materials and Methods

Molecular biology

All general reagents were obtained from Fisher Scientific (Loughborough, U.K.) or Sigma Aldrich (Poole, U.K.) unless otherwise stated. The porcine muscle Uni-ZAP XR cDNA library was obtained from Stratagene (La Jolla, CA). UltraSpec RNA isolation medium was purchased from Biotecx (Houston, TX). Promega (Southampton, U.K.). RNase inhibitor rRnasin and pGEM-T vector kit were obtained from Promega (Southampton, U.K.). The GeneClean II DNA purification kit was obtained from Anachem (Luton, U.K.). Plasmid and mRNA purification kits were purchased from Qiagen (Milwaukee, WI). Deoxy-NTPs were purchased from Boehringer (Mannheim, Germany). DNA primers. The presence of cDNA encoding the transmembrane and GPI-anchored forms of pig DAF was generated by PCR amplification from pBluescript M15799. The fragment was isolated from an agarose gel and labeled with [α-32P]dCTP using the RediPrime kit (Amersham) according to the manufacturer’s protocol. The labeled probe was purified and used to screen 600,000 plaque colonies of the porcine aortic smooth muscle (Novartis, Horsham, U.K.). Mouse mAb anti-human DAF (BRIIC16) was obtained from International Blood Group Reference Laboratories (Oxford, U.K.). Mouse mAb anti-pig MCP (6C5) was raised as previously described (23).

Screening of a pig cDNA library

A DNA fragment encompassing nucleotides +26 to +1131 (aa residues +26 to +345) of the human DAF cDNA sequence (GenBank accession no. M15799) was generated by a Sst1-Ncol digest of the expression vector pDR2DAF (25). The fragment was isolated from an agarose gel and labeled with [α-32P]dCTP using the RediPrime kit (Amersham) according to the manufacturer’s protocol. The labeled probe was purified and used to screen 600,000 plaque colonies of the porcine aortic smooth muscle. The DNA library plated at 50,000 PFU/plate and grown on a lawn of XL1-Blue Escherichia coli for 8 h. Duplicate lifts were taken onto Hybond-N nylon membranes, denatured, UV cross-linked, and incubated with 32P-labeled probe (18 h at 45°C). Membranes were washed and exposed to x-ray film.

Plaques positive on both duplicate membranes were identified, isolated in Agar plugs, eluted in 1 ml of SM buffer (100 mM NaCl, 8 mM MgSO4, 50 mM Tris-HCl, 0.01% eluate in pH 7.5) for 24 h, and replated. The above screening protocol was then repeated. Individual positive plaques from the secondary screening were isolated in agar plugs and eluted in SM buffer. Putative positive clones were confirmed by PCR and Southern blotting. PCR was achieved using primer pairs (23) that amplify transmembrane (AAG AGC TAT GAC GAT G) and reverse (AAG ATC TAT GAC GAT G) domains. The probe was purified and used to screen 600,000 plaque colonies of the porcine aortic smooth muscle. The DNA library plated at 50,000 PFU/plate and grown on a lawn of XL1-Blue Escherichia coli for 8 h. Duplicate lifts were taken onto Hybond-N nylon membranes, denatured, UV cross-linked, and incubated with 32P-labeled probe (18 h at 45°C). Membranes were washed and exposed to x-ray film.

Northern blotting, RT-PCR analysis, and Southern blotting

Total RNA was isolated from fresh pig tissues and cells using Ultraspec RNA isolation reagents (Biotecx) following the manufacturer’s instructions. mRNA was isolated from pig cell lines using the Oligotex mRNA isolation kit (Qiagen). A DNA fragment encompassing a SCR–1–3 of pig DAF was generated by PCR amplification from pBluescript vector containing pig DAF sequence. The PCR used primers N5 (GAC TGC AGC CTT CCA GCC GA) and N7 (ATC ACT CCA CTC AAC ATC ATT T) with the following reaction conditions: 94°C for 30 s, 61°C for 30 s, 56°C for 1 min (ramp 1.0), and 72°C for 3 min for 25 cycles. The PCR products were resolved on agarose gels, transferred to Hybond-N, and probed using the human DAF probe described above. The CDNA inserts were recovered from PCR screen-positive colonies using the Exassist/ SOLR system (Stratagene, La Jolla, CA). Individual bacterial colonies containing combined phagemid pools were grown up inuria Bertani broth containing 50 μg/ml kanamycin, and phagemid DNA was purified using a QIAprep spin plasmid mini-prep kit (Qiagen, Valencia, CA). Automated sequencing was conducted in-house using an ABI model 377 DNA sequence (Applied Biosystems, Warrington, U.K.). Sequences were analyzed using PSORT-II software (http://psort.iim.u-tokyo.ac.jp/).

Tissues, cells, and sera

Pig blood was obtained fresh from the animal facility of the University of Wales College of Medicine or from a local abattoir, either without anti-coagulant for collection of serum or with EDTA (20 mM final concentration) for cells and plasma. Serum was harvested and stored in aliquots at −70°C. Human serum was obtained from normal donors. Pig erythrocytes were separated from EDTA blood by centrifugation, careful removal of buffy coat, and washing in PBS as described previously (23). Pig leukocytes (total and separated granulocytes and mononuclear cells) were obtained by centrifugation of EDTA blood over Ficoll by standard methods as previously described (23). Pig tissues were obtained fresh from the facilities noted above and processed immediately for mRNA isolation. The pig cell lines IB-RS-2 (kidney) and ST (testis) were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were propagated in DMEM supplemented with 10% FCS, glutamine, pyruvate, essential amino acids, and penicillin/streptomycin.

Abs and proteins

Mouse mAb anti-human C3 (C3/30) was a gift from Dr. Peter Taylor (Novartis, Horsham, U.K.). Rabbit polyclonal anti-pig C3 antisera was generated in in-house by immunization with purified pig C3 in adjuvant. Rabbit and guinea pig anti-Chinese hamster ovary (CHO) cell antisera were generated by repeated immunization with cells in pertussis vaccine. FITC- and HRP-conjugated secondary Abs against mouse and rabbit Ig were obtained from Bio-Rad (Hemel Hempstead, U.K.). PE-conjugated secondary Abs against mouse and rabbit Ig were obtained from Dako (Hobart, U.K.). Hybrid Ig were obtained from Bio-Rad (Hemel Hempstead, U.K.), and Sigma Aldrich, respectively. Mouse mAb anti-pig CD59 (MEL-2 and MEL-3) and anti-human DAF (MBC1) were raised in this laboratory (24, 26). Mouse mAb anti-human DAF (BRIIC16) was obtained from International Blood Group Reference Laboratories (Oxford, U.K.). Mouse mAb anti-pig MCP (6C5) was raised as previously described (23).

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for 30 s, and 72°C for 30 s for 25 cycles. The PCR products obtained were resolved on a 1% agarose gel, blotted onto Hybond-N membranes, and subjected to denaturation and UV cross-linking as described above.

To generate a probe for Southern blotting of PCR products, a 459-bp DNA fragment was PCR amplified using primers N1 and TM-AS as described above and was purified by agarose gel electrophoresis. The purified DNA fragment was labeled with alkaline phosphatase (AlkPhos Direct, Amersham Pharmacia), following the manufacturer’s protocol. Membranes were hybridized with the probe and washed, and the signal was detected using CDP star detection reagent (Amersham Pharmacia). The positions of all primers are shown in Fig. 1.

**FIGURE 1.** Nucleotide and deduced amino acid sequence of pig DAF. The 1546-bp sequence obtained from library screening is shown. The initiation codon (atg) is underlined. Primers used in RT-PCR and sequencing are overscored with arrows and named. The polyadenylation site in the 3’-untranslated sequence (ataaa) is underlined. The 176-bp sequence spliced out to generate the GPI-anchored form is boxed in gray, and the new carboxyl terminus is shown following this. The first amino acid of the mature protein (D) is double underlined. Numbering is for the mature protein sequence; double numbering (304/259) indicates the end of the tm isoform and continuation of gpi isoform in that line. Potential N-glycosylation sites (NXS/T) are indicated by C. Arrows indicate the predicted sites of GPI anchor addition in isoforms of pDAFgpi with and without ST-c sequence. GenBank accession numbers are AF228059, AF228060, and AF228061.
25 pmol of primers Q0 and N1 with the following reaction conditions: 94°C for 30 s, 64°C for 30 s (ramp 1.0), and 72°C for 3 min for 25 cycles. In the second amplification a 3-μl aliquot of a 1/20 dilution of the first reaction was amplified using 25 pmol of Q1 and 25 pmol of N2 with the following reaction conditions: 94°C for 30 s, 61°C for 30 s (ramp 1.0), and 72°C for 2 min for 25 cycles. The PCR products were resolved on agarose gels, purified, and sequenced as described above. The positions of all primers are shown in Fig. 1.

Generation of a pig DAF-Ig fusion protein and production of mAbs
cDNA encoding the three SCRs of pig DAF was cloned into the expression vector Signal plg Plus (R&D Systems, Abingdon, U.K.) according to the manufacturer’s instructions. The cloning site was located just upstream from DNA encoding the hinge and Fe regions of human IgG1. DNA encoding DAF, Ab hinge, and Fe was amplified by PCR using purified vector as template and was sequenced to confirm fidelity. Primers incorporated restriction sites enabling subsequent ligation into the expression vector pDR2AEpI. CHO cells were transfected with vector using Lipofectamine (Life Technologies, Gaithersburg, MD) as described previously (24). A stable line was established by selection with hygromycin B, and culture supernatant containing pig DAF-Ig fusion protein (pDAF-Ig) was collected. The fusion protein was purified by protein A affinity chromatography (Prosep A, Bioprocessing, Consett, U.K.) and was used to immunize mice. Hybridomas were generated using established protocols. Supernatants were screened by ELISA on plates coated with the pig DAF-Ig fusion protein or an irrelevant Ig-fusion protein as a negative control. Positive clones were recloned to obtain monoclonal populations.

Expression of pig DAF in CHO cells
Insert containing full-length cDNA encoding transmembrane pig DAF was released from the pBluescript vector by digestion with enzymes Xbal and Nhel and was purified by agarose gel electrophoresis. Full-length cDNA encoding GPI-anchored DAF was obtained by RT-PCR from granulocyte mRNA using VENT polymerase (New England Biolabs, Hitchin, U.K.) and primers PDAF-Xba (GGT TCT AGA GCG GTG AGG CGC CTA ATG GCC) and PDAF-GPI (GGT GGA CCT TCT TGG CTA AGT CAG CTA GCC). The primers contained, respectively, Xbal and BamHI restriction sites. The PCR product was digested with these enzymes. Each of the cDNAs obtained was ligated into pDR2AEpI previously digested with Xbal and BamHI. After electroporation into DH5α, colonies were picked, and the plasmids were purified. The fidelity and orientation of each of the pig DAF cDNA in the vector were confirmed by sequencing. CHO cells were Lipofectamine transfected with DAF-containing vector, with empty expression vector (negative control), or with expression vector containing human DAF cDNA as described previously (24). Surface expression and the nature of the membrane anchor were demonstrated by flow cytometry as described below. Expression was confirmed by SDS-PAGE and Western blot analysis using anti-pig DAF Ab. Cells were transfected with pig erythrocytes, leukocytes, granulocytes, and platelets in a similar manner.

Flow cytometry
Cells were harvested, washed three times with PBS, and resuspended at 10^6/ml in FACS buffer (1% BSA and 0.2% NaN₃ in PBS). All staining steps were conducted on ice. Cells were incubated with primary Ab (2–5 μg/ml) for 30 min, washed three times with FACS buffer, and incubated for 30 min with a PE- or FITC-conjugated secondary Ab. Cells were washed three times in FACS buffer and fixed with 1% formaldehyde in FACS buffer. Fluorescence was measured on a Becton Dickinson FACScalibur (San Jose, CA).

Susceptibility to cleavage by PIPLC was assessed by incubation of cells (2 × 10^6/ml in PBS) at 37°C for 30 min with PIPLC (0.14 U/ml final concentration) at 4°C. Cells were washed, stained, as described above with mAb MBC-PD1 (anti-pig DAF), BRC126 (anti-human DAF), JM4C8 (anti-pig MCP), or MEL-2 (anti-pig CD59), and analyzed by flow cytometry.

C3 deposition assay
CHO cells transfected with transmembrane pig DAF, human DAF, or vector control were harvested by incubation in PBS, 20 mM EDTA, and 1% (w/v) BSA. Cells (10^6/ml) were incubated for 30 min at 4°C with 10% (v/v) rabbit anti-CHO antiserum (for activation of human C) or 0.7% (v/v) guinea pig anti-CHO antiserum (for activation of pig C). Both anti-CHO antiseras were heat-treated (56°C, 30 min) to inactivate C before use. Cells were washed twice in fixation diluent (CFD; Oxoid, Basingstoke, U.K.) containing 1% BSA, resuspended at 10^6/ml, and incubated with an equal volume of a dilution in CFD of either C8-depleted human serum or normal pig serum for 25 min at 37°C. Cell viability was monitored by trypan blue exclusion. Cells were washed twice in FACS buffer and stained for C3 deposition using mAb anti-human C3 (C3-30; 2 μg/ml) for detection of human C3 fragments or rabbit polyclonal anti-pig C3 (1/250 dilution) for detection of pig C3, followed by either rabbit anti-mouse Ig-FITC (Dako) or goat anti-rabbit Ig-FITC (Harlan Seralab, Crawley Down, U.K.). C-activating antisera were selected for negligible cross-reactivity with these secondary Abs. C3 deposition was measured by flow cytometry.

Calcine release assay
CHO cells were seeded into 24-well plates 48 h before the assay. Hygromycin B was omitted from the culture medium, and cell density was adjusted to yield approximately 70% confluence at the time of assay. Cells were incubated at 37°C for 30 min with 200 μl of calf-ALM (Molecular Probes, Portland, OR) at 2 μg/ml in culture medium containing 5% FCS. Calcine-loaded cells were rinsed once in ice-cold CFD/1% BSA and incubated at 4°C for 30 min in 175 μl of the same buffer containing 10% heat-inactivated rabbit anti-CHO antiserum (C3-30), 6C5 (function-blocking anti-MCP) (23), and PD2 (anti-pig DAF, nonfunction blocking). Calcine remaining in cells was released by incubation for 15 min with 200 μl of 0.1% Triton X-100 in CFD/1% BSA. Calcine release by serum or detergent was measured with a Wallac fluorometer (Life Sciences International, Basingstoke, U.K.) with excitation at 485 nm and emission at 530 nm. The percent calcine released by serum was calculated as follows: % release = 100 × calcine released by serum/(calcine released by serum + calcine released by detergent). Background release was assessed by incubation with buffer only. For function blocking studies, 20 μg/ml MBC-PD3 function-blocking anti-DAF mAb or MBC-PD1 nonblocking control mAb (both IgG1 isotype) was included with the sensitizing Ab.

Function blocking studies on pig erythrocytes

Classical pathway. Pig E were sensitized by incubation of E (2% (v/v) in PBS) for 30 min at 37°C with a 1/20 dilution of a cross-reactive rabbit polyclonal antiserum raised against pig aortic endothelial cells (prepared in-house). Ab-sensitized E were washed in CFD/1% BSA and resuspended at 1% (v/v). AB-sensitized E (50 μl) was incubated for 20 min at 37°C with 50 μl of CFD/BSA containing MEL3 (function-blocking anti-pig CD59) (24), 6C5 (function-blocking anti-MCP) (23), and PD2 (anti-pig DAF, nonfunction blocking), PD3 (anti-pig DAF, function blocking), or an irrelevant isotype control Ab (20 μg/ml of each Ab). Pig or human serum (50 μl of an appropriate dilution) was added to the cells, mixed, and incubated for 45 min at 37°C. Cells were pelleted by centrifugation, and hemoglobin release was assessed by absorbance of the supernatant at 415 nm. Background release (0%) and 100% release were measured by incubating cells with buffer only or with 0.05% Triton X-100, respectively. Background absorbance of each serum dilution was also measured: % lysis = 100 × (A415 sample − A415 serum) / (A415 100% control − A415 0%).

Alternative pathway. Pig E (10% (v/v) in 50 mM sodium acetate (pH 5.5), 150 mM NaCl, 0.1% CaCl₂, 2H₂O) were incubated with neuraminidase (0.1 U/ml; Roche) for 1 h at 37°C. Cells were washed and resuspended at 1% (v/v) in alternative pathway buffer (5 mM sodium barbitone, pH 7.4, containing 150 mM NaCl, 7 mM MgCl₂, 10 mM EDTA, and 1% BSA). The assay setup and calculations were identical with those described above, except that MEL-2 was used to block the function of CD59.

Results
Isolation and characterization of pig DAF cDNA
Screening of a porcine muscle cDNA library identified a single positive clone from a total of 6 × 10⁶ screened. The clone comprised 1546 bp and contained a single open reading frame encoding a 37-aa signal peptide, a 187-aa region containing three SCR domains, a 61-aa ST-rich region, a 19-aa sequence compatible with a transmembrane anchor, and a 37-aa putative cytoplasmic tail (Fig. 1). Comparison with the sequence of human DAF revealed amino acid identity of 64% through the three SCR (67% for SCR1, 67% for SCR2, 57% for SCR3), with all Cys residues conserved between species (Fig. 2). These data strongly suggested that the cDNA isolated was a pig analog of DAF. No sequence resembling SCR4 in human DAF was found. The first 20 aa of the
ST-rich region were 55% identical with the first 20 aa of ST-a in human DAF. The next 34 aa of the ST-rich sequence were 39% identical with sequence in ST-b of human DAF. The final 7 aa of the ST-rich sequence were 57% identical with ST-c in human DAF. The putative transmembrane segment was 42% identical with the transmembrane region in guinea pig DAF. The cytoplasmic domain also had some homology with that in guinea pig DAF (38%). This isoform is henceforth referred to as pDAFtm.

Sequencing from granulocyte RNA following 3' RACE identified a second cDNA of 1370 bp, identical with the original cDNA through the first 929 bp but with the subsequent 176 bp deleted. This encoded an isoform of pig DAF identical with pDAFtm through the SCRs and ST-rich region to the end of ST-c, but with a new carboxyl terminus generated by the frame shift comprising 17 aa (Fig. 1). This new sequence was 56% identical with the carboxyl terminus of human DAF and provided a putative GPI anchor addition signal; this isoform is henceforth termed pDAF-gpi. The predicted site of GPI anchor addition, based upon the consensus GPI signal and comparison with human DAF, is at S241. Another cDNA isolated from granulocytes was identical with that described above, except that the 21-bp sequence encoding ST-c was deleted. The GPI anchor addition consensus signal was retained in this isoform with the predicted anchor addition site at S234. All DAF isoforms contained three potential N-glycosylation sites, the first at the junction of SCR1 and SCR2 (N60), the second in SCR2 (N113), and the third in SCR3 (N140). Only the first of these is present in human DAF.

**Pig DAF mRNA is heterogeneous and broadly expressed in tissues**

To estimate the size of pig DAF mRNA, total RNA freshly extracted from pig spleen, liver, and testis was analyzed by Northern blotting. The probe used contained sequence corresponding to SCR1–3 of pig DAF and should detect all forms of pig DAF. As shown in Fig. 3A, multiple bands were detected in each of the tissues. The major bands were present at approximately 3.3, 2.8, and 1.6 kb. These same bands were present in mRNA isolated from pig cell lines (Fig. 3B). The GAPDH probe gave similar intensity of staining in each tissue sample (not shown).

To characterize the respective tissue distributions of pDAFtm and pDAFgpi, RT-PCR was performed on mRNA from various tissues using a primer specific for SCR3 and either a primer specific for the unique sequence in pDAFtm to amplify only transmembrane forms (Fig. 4A) or a primer from a portion of the 3'-untranslated region common to both transmembrane and GPI isoforms (Fig. 4B). The predicted length of the product for pDAFtm in the first reaction was 458 bp. The predicted lengths of the products for pDAFtm and pDAFgpi in the second reaction...
were 571 and 395 bp, respectively. The pDAFtm-specific reaction (Fig. 4A) demonstrated that mRNA encoding pDAFtm was widely distributed and was present in all tissues tested, albeit very weakly in granulocytes. In contrast, pDAFgpi expression was abundant in granulocytes and also in lung, but was essentially absent from testis and kidney (Fig. 4B). In each reaction the major products were of the predicted size, but several larger products were obtained. Southern blotting of the gels using a probe specific for the common ST region demonstrated that all the bands obtained were derived from pig DAF mRNAs (data not shown). Additional large bands (1 and 1.2 kbp) were particularly abundant in granulocytes, and these products were sequenced. None contained sequence suggestive of a fourth SCR. Instead, each contained 606 bp of additional ST-a sequence encoding 202 aa, including 10 copies of a 17-residue repeating unit (Fig. 5). A 34-aa homology unit was also apparent in this novel ST sequence, each pair of 17 residue repeats being highly homologous with the next (Fig. 5). The larger band (1.2 kbp) encoded a transmembrane-anchored isoform, and the smaller (1 kbp) encoded a GPI-anchored isoform.

In an effort to find pig DAF transcripts containing a fourth SCR, all products obtained from PCR and 3’-RACE from the various tissues and cell lines were sequenced directly from gels and/or following a cloning step. Products obtained using different primer sets spanning the SCR3-ST junction were also sequenced. In all these analyses, no sequence suggestive of a fourth SCR was found. All sequences resembled those described above, differing only in membrane anchoring and length of the ST region.

Pig DAF protein is expressed on all circulating cells, but anchoring differs between cell types

None of the available mAb against human, rat, or mouse DAF stained pig cells. mAb specific for pig DAF were therefore generated using a pig DAF-Ig fusion protein as immunogen. Several mAb were obtained, three of which (MBC-PD1, MBC-PD2, and MBC-PD3; all IgG1 isotype), were shown by flow cytometry to stain pig cells and CHO cells transfected with cDNA encoding pDAFtm. The expression of pig DAF was strong on erythrocytes, and treatment with PIPLC failed to release the protein, indicating that erythrocyte pig DAF was transmembrane anchored (Fig. 6). As a control, the same dose of PIPLC was shown to efficiently release GPI-anchored pig CD59 from pig erythrocytes (Fig. 6). The transmembrane nature of pig erythrocyte DAF was further confirmed by demonstrating that the purified protein failed to incorporate into sheep erythrocytes (data not included). Unfractionated PBMC gave a broad staining distribution for pig DAF, and PIPLC treatment caused a small reduction in expression. In contrast, isolated granulocytes strongly expressed DAF, and treatment with PIPLC reduced expression by almost 90% (Fig. 6).

Cells were also analyzed for DAF expression by SDS-PAGE under nonreducing conditions and Western blotting (Fig. 7). From erythrocyte lysates, pig DAF ran as a broad band of approximately 45–52 kDa; from lysates of mononuclear cells and platelets, pig DAF gave a sharp band with an apparent $M_r$ of 52 kDa; granulocyte lysates gave a broad band of 45–50 kDa. High $M_r$ bands were apparent, particularly in erythrocytes and platelets, which presumably represent DAF aggregates. None of the mAb recognized pig DAF following reduction (data not shown).
Expression of pig DAF in CHO cells

Stable populations of CHO cells expressing pDAFtm, pDAFgpi, the ST-c-deleted isoform of pDAFgpi, or human DAF were generated. The pDR2 vector was chosen because it is reported to give reproducible high levels of expression of different cDNAs in a given cell type (29, 30). It was therefore anticipated that similar levels of expression of human and pig DAF would be achieved. Expression was assessed by flow cytometry and Western blotting as described above, using BRIC216 anti-human DAF and MBC-PD1 anti-pig DAF. Uniformly high levels of expression were obtained for all proteins (Table I). Neither of the mAb recognized vector control cells. BRIC216 was negative on pig DAF-transfected cells, and MBC-PD1 was negative on human DAF-transfected cells. Although precise comparison of expression level based upon staining with different reagents is not possible, the data suggested that human DAF and the different pig DAF isoforms were expressed at similar levels (Table I). The level of expression of pig DAFtm in the transfected CHO cells was 14-fold that of endogenous DAF on pig erythrocytes, as assessed by flow cytometry (data not shown). Treatment of human DAF transfectants with

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Table I. Expression of pig DAF isoforms on CHO cells

<table>
<thead>
<tr>
<th>Isotype control (6D1)</th>
<th>No PIPLC (MBC-PD1 or BRIC216)</th>
<th>PIPLC treated (PD1/BRIC216)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human DAF</td>
<td>2.3 ± 0.58</td>
<td>897 ± 27</td>
</tr>
<tr>
<td>pDAFtm</td>
<td>2.9 ± 0.14</td>
<td>658 ± 46</td>
</tr>
<tr>
<td>pDAFgpi</td>
<td>4.0 ± 1.7</td>
<td>409 ± 16</td>
</tr>
<tr>
<td>PDAFgpi (no ST-c)</td>
<td>3.0 ± 1.0</td>
<td>511 ± 27</td>
</tr>
</tbody>
</table>

CHO cells were transfected with the various isoforms of pig DAF or with human DAF. The stable transfectants obtained were assessed for expression level by staining with the appropriate mAb, MBC-PD1 anti-pig DAF, or BRIC216 anti-human DAF and analyzed by flow cytometry. Cells were also treated with PIPLC to release GPI-anchored proteins before staining with mAb. Results are means of triplicate tubes ± SD.
PIPLC decreased the mean cell fluorescence by 75%; PIPLC treatment caused no reduction in expression of pig DAFtm, but caused a 65–75% decrease in expression for each of the pDAFgpi isoforms, confirming the predicted modes of anchoring (Table I).

Western blotting of CHO-pDAFtm cell lysates under nonreducing conditions (Fig. 7) identified a major band at approximately 55 kDa and a minor band, presumably unglycosylated protein, at 36 kDa. Blotting of CHO expressing each of the pDAFgpi isoforms gave a band of approximately 50 kDa (data not shown).

**Functional analysis of pDAFtm**

Protection against C was assessed in transfected CHO cells by measurement of C3b deposition and inhibition of C lysis. Deposition of either pig or human C3b on CHO cells transfected with pDAFtm was measured by flow cytometry and compared with C3b deposition on human DAF-transfected CHO cells and cells transfected with vector alone as a control. Following incubation with pig serum, high levels of C3b were detected on vector control cells whereas cells expressing human DAF bound almost no C3b, even at the highest serum doses (Fig. 8A). Cells expressing pDAFtm did not differ significantly from vector control cells, suggesting that pDAFtm had little or no effect on accumulation of pig C3b. Following incubation with human serum, human DAF again efficiently prevented accumulation of C3b compared with vector controls (Fig. 8B). Expression of pDAFtm also significantly reduced accumulation of human C3b, albeit less efficiently than human DAF (Fig. 8B).

Protection from C lysis by pDAFtm expressed on CHO cells was evaluated and compared in a calcein release assay with that conferred by expressed human DAF. Transfectants expressing pDAFtm, human DAF, or vector alone were sensitized and incubated with either pig or human serum at different dilutions. Both...
sera readily lysed the sensitized vector control cells, achieving approximately 85% lysis for human serum and 80% for pig serum at a 1/4 dilution (Fig. 9). Expressed human DAF markedly inhibited lysis by human and pig C, whereas expressed pig DAF inhibited human C, albeit less efficiently, but had negligible activity against pig C (Fig. 9). To confirm that the observed lytic protection against human C was DAF mediated, pDAFtm-expressing cells were preincubated with the function-blocking anti-pig DAF mAb MBC-PD3 or the nonblocking mAb MBC-PD1. Preincubation with the function-blocking mAb eliminated the observed protective effect against human C, whereas incubation with the isotype-matched nonblocker did not (Fig. 9).

To further explore the C regulatory capacity of pDAFtm, pig E were sensitized with rabbit antiserum for CP activation or treated with neuraminidase for AP activation. Incubation of pig EA with the function-blocking anti-pig DAF mAb MBC-PD3 caused enhanced lysis by human serum but not by pig serum, whereas the nonblocking mAb had no effect (Fig. 10, A and B). These data support the results obtained in CHO transfectants. Under AP conditions, blocking pig DAF had no effect on lysis of pig E by either pig or human serum (Fig. 10, C and D), indicating that pig DAF does not function as an AP regulator.

Discussion
DAF has a long history, dating back to the first description of its activity in erythrocyte stroma 30 years ago (31, 32). Despite this lengthy history, new information on this fascinating molecule continues to emerge. Apart from its role as a membrane regulator of C, it is now apparent that DAF has roles as receptor for viruses and other micro-organisms and as ligand for activated leukocytes (33–35). The characterization of DAF analogues in primates and rodents has shown that there is conservation of both structure and function across species (14, 15, 17, 36). In other species alternative splicing generates several different isoforms of DAF; in the mouse two separate genes exist, each encoding multiple forms of DAF (18). All DAF analogues identified to date comprise, from the amino terminus, four SCR domains, a heavily O-glycosylated Ser/Pro/Thr-rich (ST) region of variable length, and a membrane attachment that, in the majority of cases, is a GPI anchor. Transmembrane (tm) forms of DAF have been described in mouse and guinea pig, and secreted forms have been reported in human, rat, mouse, and guinea pig, but all have in common the four SCR domains. Domain deletion studies in human DAF have shown that SCR2 and -3 are essential for C regulatory activity (6). SCR1 appears to play no role, and the importance of SCR4 is controversial. The ligand binding site has recently been localized to a cavity in SCR2 and a portion of the groove between SCR2 and SCR3 (8). Three tandem Lys residues (K125-K127) at the bottom of this groove are implicated in ligand binding.

We here describe the identification and characterization of the pig analogue of human DAF. Several isoforms of pig DAF were identified, both tm and GPI anchored and with variability in the length of the ST region. All isoforms of pDAF contained only three SCR domains, homologous with the first three SCRs in human DAF. The ligand binding site described above, comprising three tandem Lys residues in the groove between SCR2 and SCR3 (8), is well conserved in pig DAF. Several approaches, including direct sequencing of cell and tissue-derived mRNA, and RT-PCR or 3′-RACE followed by sequencing, were used in an attempt to find a DAF isoform containing a fourth SCR in a large panel of cells and tissues and from different strains of pig (large white and laboratory mini-pigs). No
such sequence was identified. From these data we conclude that DAF in pigs is unique in that it lacks SCR4. The process by which SCR4 has been lost is not yet known. In man SCR4 is encoded in a single exon, exon 6 of the Daf gene. Two possibilities exist to explain the loss of SCR4 in pigs, either the coding exon has been lost from the gene or it is present but not transcribed. Screening of a pig genomic library with a human SCR4-derived probe has yielded negative results. Current efforts are focussed on sequencing between exons encoding the carboxyl-terminal portion of SCR3 and ST-a in genomic clones obtained from the library, a distance in excess of 11 kbp.

The longest ST region, obtained from granulocyte RNA, contained an insert of 202 aa of additional ST-a sequence, composed almost entirely of 10 repeats of a 17-aa unit homologous with sequence in ST-a of human DAF (Fig. 5). A second homology unit comprised sequential pairs of the 17-mer repeats, yielding five highly homologous 34-aa repeats. A similar 17-aa ST repeating unit was described in guinea pig DAF and was shown to be responsible for variation in ST region length (14). The tm region in pDAFtm was homologous with the tm regions in guinea pig and mouse tm DAF. Of note, the predicted membrane-spanning region in pig DAF includes the last four amino acids of ST-c. The cytoplasmic region was homologous with that in guinea pig DAF and contained a consensus ATP/GTP binding site (P-loop) motif (GFWHYGKS), the significance of which is unknown (Fig. 2) (15, 36).

Northern and RT-PCR analysis of RNA extracted from pig tissues and cell lines indicated that pig DAF was widely expressed, although the expression levels varied between tissues (Figs. 3 and 4). These analyses also identified the presence of multiple isoforms of DAF, differentially expressed in the various tissues. To identify the expression of the major isoforms in tissues and cells, RT-PCR was performed using primers specific for pDAFtm or capable of amplifying all pDAF isoforms. These studies demonstrated that pDAFtm was broadly expressed, whereas pDAFgpi was significantly expressed only in granulocytes and lung (Fig. 4). Granulocytes expressed predominantly pDAFgpi.

Flow cytometric analyses using mAb generated against a pDAF-Ig fusion protein confirmed the abundant expression of pDAF on circulating cells (Fig. 6). Pig erythrocyte DAF was not released by PIPLC treatment, although pig CD59 on the same cells was efficiently released. The transmembrane anchoring of DAF on pig erythrocytes was confirmed by immunoaffinity purification from erythrocytes, demonstrating that the protein did not incorporate into guinea pig erythrocytes, a property of the GPI anchor in human DAF. In sharp contrast, treatment of granulocytes with PIPLC efficiently released pDAF, as predicted from the mRNA analyses. Mononuclear cells expressed predominantly pDAFtm. This result parallels the situation described for FcγRIII (CD16) in humans. GPI-anchored CD16 is expressed by granulocytes, whereas a tm form is expressed by macrophages and NK cells (37). Early studies on mouse DAF suggested that the protein on erythrocytes was tm anchored (15). However, recent studies contradict this; mice in which the gene encoding GPI-anchored DAF has been deleted apparently lack DAF on erythrocytes, which are consequently susceptible to C lysis (38).

The most abundant form of pig DAF thus differs from human DAF in two important respects: it is tm anchored, and it lacks SCR4. Transmembrane-anchored forms of human DAF have been engineered and expressed, and C regulatory activity compared with that of GPI DAF (39). Both forms were equally effective at inhibiting C, making it unlikely that tm anchoring adversely affects function in pig DAF. The degree to which deletion of SCR4 in human DAF compromises C regulatory activity is unclear. One study reported that human DAF lacking SCR4 had no C inhibitory activity when expressed on CHO cells (6). A more recent study in which DAF lacking SCR4 was examined as a soluble protein or after incorporation into erythrocytes described inhibition of the classical, but not the alternative, activation pathway (7). To assess the capacity of pDAFtm to regulate C we generated stable transfectants in CHO cells abundantly expressing the molecule. CHO cells expressing either pig or human DAF showed a single homogeneous population with high expression of the regulator. Transmembrane anchoring of the expressed pDAFtm was confirmed by the failure of PIPLC to cause release. Cells were compared in assays of classical pathway C regulation. In C3b deposition assays, pDAFtm inhibited human C, albeit less efficiently than human DAF expressed in the same system, but had no significant activity against pig C (Fig. 8). In lytic assays, pDAFtm inhibited human C, but again had a negligible influence on pig C lysis, whereas human DAF was strongly inhibitory for both human and pig C (Fig. 9). A blocking anti-pig DAF mAb reversed protection conferred by expressed pig DAF.

To confirm these findings in a different setting, the effect upon lytic susceptibility of blocking DAF on pig erythrocytes was assessed. The blocking mAb enhanced CP lysis by human serum, but not by pig serum; a control nonblocker had no effect. These data confirm that pDAFtm, the sole isoform expressed on pig erythrocytes and the major isoform expressed on most other cells, can inhibit human C, but has no significant regulatory activity against pig C when tested in vitro in a classical pathway assay. These findings have been replicated in a classical pathway hemolysis assay using soluble recombinant pig DAF; regulation of human, but not pig, C was obtained (data not included). Treatment of pig E with neuraminidase rendered the cells activating for the AP. Blocking of pig DAF with mAb had no effect on lysis under AP conditions regardless of the serum source, indicating that pig DAF does not regulate the AP of pig or human C. These data are not surprising, in that others have reported that SCR4 is essential for AP regulatory activity of human DAF (7).

The demonstration that pig DAF does not significantly regulate pig C is most unexpected. Pig erythrocytes, unlike human erythrocytes, abundantly express MCP, and MCP is an important C regulator on these cells (23). It is therefore possible that pig erythrocytes and other plasma-exposed cells are adequately protected from C activation by MCP. An alternative possibility is that pigs might express another membrane regulator, perhaps a molecule analogous to Cry in rodents. Further, it remains possible that other isoforms of pig DAF may retain regulatory capacity for pig C. We are currently examining C regulatory function in pDAFgpi and in isoforms with different lengths of ST. Guinea pig DAF isoforms with long ST regions were better regulators of C (40), and it has previously been reported that deletion of the ST region in human DAF ablates C regulatory activity (6). The role of the ST region is to act as a spacer to place the active sites contained within theSCRs at an appropriate distance from the membrane. It is possible that the long ST isoforms of pig DAF will show increased pig C regulatory activity. We are also engineering pDAFtm to express a fourth SCR to examine whether this confers pig C regulatory capacity on the molecule.

What, then, is the role of DAF on pig erythrocytes? One may speculate that pig DAF serves roles other than C regulation on pig erythrocytes and other circulating cells, possibly serving as a C receptor with roles in handling immune complexes. In man this role is served by CR1, and no analogue of CR1 has yet been discovered in the pig. Pig DAF may have evolved to fill this role and, in the process, lost the capacity for intrinsic C regulation. The long ST isoforms, projecting the SCRs far from the membrane, might
be particularly relevant to immune complex handling. Experiments to test this hypothesis are currently in progress.

It remains puzzling that pigs alone of all the species studied to date have lost SCR4 in DAF. In humans, SCR4 is implicated in the binding of E. coli adhesins and enteroviruses (33, 34). It is possible that loss of SCR4 in pigs has been selected in evolution, because it renders cells resistant to infection with these pathogens. Our preliminary data support this hypothesis in that echoviruses that use human DAF as receptor cannot bind pig DAF. Precedent exists in that MCP, which contains a measles virus binding site in SCR1, is expressed in New World monkeys as a three SCR molecule lacking SCR1 and unable to bind measles virus (41). A concern emerging from these studies is that pig organs engineered to hyperexpress human DAF for use in xenotransplantation (42) might be rendered susceptible to infection by these common human pathogens with potentially disastrous consequences.

Acknowledgments

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References