Molecular and functional analysis of mouse decay accelerating factor (CD55)

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Molecular cloning of mouse decay accelerating factor (DAF; CD55) predicted two forms of the molecule, one transmembrane (TM) and the other glycosylphosphatidylinositol (GPI)-anchored; these are encoded by separate genes termed *Daf-GPI* and *Daf-TM*. In the present study several additional isoforms of mouse DAF, generated by alternative splicing from these genes, are described. Northern-blot analysis of RNA and reverse transcriptase-PCR from various tissues indicated that spleen and testis expressed high levels of DAF, which comprised several species. These species were cloned and sequence analysis revealed various novel forms in addition to those previously reported. Two novel forms were derived from the *Daf-TM* gene but the transmembrane sequence defined previously was replaced by a unique GPI-anchor addition sequence; one clone also had part of the serine/threonine/proline (STP) region deleted. A third clone, encoding a transmembrane protein, was also derived from this gene but the entire STP region was deleted. A fourth clone, derived from the *Daf-GPI* gene, contained a novel C-terminal sequence, suggestive of a secreted form of the protein. Two DAF cDNAs (TM and GPI-anchored) were stably expressed in Chinese hamster ovary cells. When these cells were attacked with mouse or rat complement and analysed for C3b deposition, DAF-transfected cells had greatly reduced C3b deposition compared with controls. Transfection with DAF also conferred protection from complement in a cell-lysis assay, and a soluble, recombinant form of mouse DAF inhibited complement in a haemolytic assay.

Key words: complement, murine, regulation.

INTRODUCTION

In the human, an array of complement regulatory molecules exist on the plasma membrane to protect cells from lysis by homologous complement. These include membrane cofactor protein (MCP; CD46), decay accelerating factor (DAF; CD55) and complement receptor 1 (CD35), which inhibit the activation pathways, and CD59, which regulates membrane attack complex formation [1]. DAF acts to accelerate decay of C3/C5 convertases, whereas MCP is a cofactor for cleavage and inactivation of C3b by the serine protease factor I. Although the functions of human MCP and DAF are different, these two molecules bear a striking similarity in the organization of their protein domains. Each has four short consensus repeats (SCRs) consisting of about 60 amino acids each, followed by a heavily glycosylated membrane-proximal serine/threonine/proline-rich (STP) region, and then either a transmembrane (TM) domain (as in MCP) or a glycosylphosphatidylinositol (GPI) anchor (as in DAF) [2–4]. The *Mcp* gene is commonly alternatively spliced, resulting in isoforms with smaller or larger STP regions and different cytoplasmic tails [5]. Although alternative splicing of the *Daf* gene has been reported, the alternative product, a putative secreted form, has not been demonstrated in plasma [3].

Regulatory molecules in other species are less well characterized but, in recent years, rapid progress has been seen in the identification of complement inhibitors in rat, mouse, guinea pig and pig. Characterization of complement regulators in the rat and mouse is crucial for analysis of their respective roles in animal models of disease, having implications for treatment of these diseases in humans. Interest in the regulatory molecules of the pig has been spurred by their potential role in xenotransplantation. In the mouse, complement receptor 1, CD59 and MCP have been cloned and shown to have comparable functions with human analogues [6–9]. Mouse cells also carry a complement inhibitor that has not been identified in humans, which has been termed Crry [7,10,11]. It appears to encompass the functions of MCP and DAF, being both a cofactor for C3b cleavage and a decay accelerator [12,13]. A similar molecule has been characterized in the rat, also known as Crry [14,15].

The first evidence of a mouse analogue of DAF was provided in 1989, when a 60 kDa protein was isolated from the membranes of mouse erythrocytes and was shown to inhibit complement activation when incorporated into the membranes of rabbit erythrocytes; this protein was suggested to be DAF but was not further characterized [16]. In 1995, a murine cDNA was identified with a 64% nucleotide and a predicted 47% amino acid identity with human DAF [17]. The protein was predicted to be GPI-anchored. Reverse transcriptase (RT)-PCR and Northern-blot analysis demonstrated that the protein was widely expressed and, although no functional data were described, it was proposed that this cDNA encoded the murine analogue of DAF. A second cDNA derived from a separate gene was also described; this cDNA encoded an alternative form of mouse DAF which had a 90% amino acid identity with the GPI-anchored form through the SCR and STP region. However, the sequence diverged substantially at the predicted site for the GPI-anchoring signal; the derived C-terminal protein sequence was characteristic of a TM protein. Northern-blot analysis and RT-PCR indicated that

Abbreviations used: STP, serine/threonine/proline-rich; MCP, membrane cofactor protein; DAF, decay accelerating factor; SCR, short consensus repeat; GPI, glycosylphosphatidylinositol; TM, transmembrane; RT, reverse transcriptase; CHO, Chinese hamster ovary; HRP, horseradish peroxidase; CFD, complement fixation diluent; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECL, enhanced chemiluminescence.

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expression of this putative TM form of DAF was limited, with preferential expression in certain tissues, notably testis and spleen. Genomic analysis indicated that the two genes lay in a head-to-tail manner, with the *Daf-GPI* gene being 5′ to the *Daf-TM* gene. Subsequent to this report, other groups had also cloned and sequenced the cDNA for mouse DAF, however only the GPI-linked form was described [18,19].

DAF has also been cloned and characterized from guinea pig and rat. Multiple forms of guinea pig DAF have been described, but in this case alternative splicing of a single gene resulted in GPI-linked, TM and secreted forms of the protein [20]. Expression of these forms of guinea-pig DAF was neither tissue nor cell specific, and both TM and GPI-anchored forms of the protein have been shown to protect cells from complement attack [21]. Two cDNAs, derived from a single gene, have been described for rat DAF, one encoding a GPI-anchored form and a second encoding a putative secreted form [22]. Both forms have been shown to protect cells from complement attack.

In the present work, the characterization of several novel cDNA clones encoding mouse *DAF* is described. We demonstrate that, in addition to the gene duplication described above, mouse *DAF* can also be alternatively spliced in much the same way as human MCP, and a part, or all, of the STP domain can be deleted. In addition, we describe alternative splicing of the *Daf-TM* gene in the testis that results in either TM or GPI-anchored forms of the protein. A novel cDNA sequence which may encode a secreted form of the protein is also described. We have expressed both TM and GPI-anchored mouse *DAF* in Chinese hamster ovary (CHO) cells and shown that expression of *Daf* by these cells leads to a decreased deposition of C3 during complement attack and protection from lysis; we have also shown that a soluble, recombinant form of mouse DAF is protective in a haemolytic assay. This is the first evidence of functional activity for mouse DAF.

MATERIALS AND METHODS

Materials

Chemicals and reagents were from Sigma (Poole, Dorset, U.K.) or Fisher Scientific (Loughborough, Leics., U.K.) unless otherwise stated below. *Taq* DNA polymerase, RNAsin and pGEM-T were from Promega (Madison, WI, U.S.A.). The vector pCR3.1 was from Invitrogen (Leek, The Netherlands), pDR2ΔEF1α was a gift from Dr I. Anegon (INSERM U437, Nantes, France) [23], and Signal pIgplus was from R&D Systems (Abingdon, Oxon., U.K.). dNTPs were from Bioline (London, U.K.) and dUTP-FITC was from Boehringer Mannheim (Lewes, East Sussex, U.K.). Primers were synthesized in-house on an Oligo 1000M DNA Synthesizer (Beckman, Palo Alto, CA, U.S.A.). Superscript RT, hygromycin B and all tissue culture reagents were from Life Technologies (Paisley, Renfrewshire, U.K.). Restriction enzymes were from Amersham (Little Chalfont, Bucks., U.K.). Prosep A was from Bioprocessing Ltd (Consett, U.K.). Polyclonal rabbit anti-FITC was from Dako (High Wycombe, Bucks., U.K.), goat anti-rabbit IgG–horseradish peroxidase (HRP) conjugate was from Bio-Rad (Hemel Hempstead, Herts., U.K.), rabbit anti-rat IgG–HRP, goat anti-rabbit IgG-R-phycoerythrin conjugate and rabbit anti-rat–FITC conjugate were from Sigma. Polyclonal rabbit anti-mouse C3, cross-reactive with rat C3, was a gift from Dr. C. van den Berg (Cardiff, U.K.). Sheep erythrocytes in Alsever's solution were from TCS Microbiology (Claydon, U.K.), and rabbit anti-sheep erythrocyte (Amboceptor) was from Behring Diagnostics GmbH (Marburg, Germany). Monoclonal and polyclonal rat anti-mouse DAF antibodies were produced in-house [24]. Soluble, recombinant human complement receptor 1 was a gift from T-Cell Sciences Inc. (Needham, MA, U.S.A.), soluble recombinant rat DAF–Fc fusion protein was produced in-house according to the procedure outlined below for mouse DAF–Fc fusion protein.

Northern blotting

Total RNA was isolated from tissues of Balb/C mice using Ultraspec Total RNA Isolation Reagent (Biotecx Laboratories Inc., Houston, TX, U.S.A.) and 15 μg was separated in a 1 % (w/v) agarose gel. Following electrophoresis, RNA was blotted using capillary action on to a Hybond N+ nylon membrane and UV-crosslinked using a Stratagene crosslinker. A DNA probe was generated by PCR from plasmid containing sequence for mouse DAF; amplification was achieved using the following primers: 5′TGCTGCTGTCCCCAACTGTA3′ (sense) and 5′ACCGACTAGCCTGTACCCTGG3′ (antisense), numbered 92–111 and 589–609 respectively in the published GPI–DAF sequence [17]. The probe was ^{32}P-labelled using a Rediprime kit (Amersham) and unincorporated nucleotides were removed using a Nick column (Pharmacia, St Albans, Herts., U.K.). The blot was hybridized overnight at 60 °C with 25 ng of the purified ^{32}P-labelled probe in 5 ml Rapid-hyb buffer (Amersham), washed once at room temperature in 2 × SSC (1 × SSC = 15 mM sodium citrate/150 mM NaCl) containing 0.1 % (w/v) SDS and twice for 5 min at 60 °C in 0.2 × SSC containing 0.1 % (w/v) SDS before exposure to photographic film. The relative amounts of RNA loaded on to the gel were assessed by rehybridizing the blot with a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Autoradiographs were scanned using FotoLook32 software with an Agfa Snapscan 600.

RT-PCR and Southern blotting

Total RNA was prepared from tissues of Balb/C mice and 1 μg was reverse transcribed according to standard protocols using Superscript RT and 27 pmol oligo(dT) (CCAGTGAGCAGAGTGACGAGGACTGGAGCTCAAGCT$_{17}$) in a total reaction volume of 20 μl. Primers used to amplify the 3′ end of GPI–DAF were 5′CAATGGAATAATGCGAGGGG3′ (sense) and 5′TATTGTATCCATTCTTCTTG3′ (antisense), numbered 711–721 and 1254–1273 respectively in the published GPI–DAF sequence [17]. The same sense primer was used to amplify the TM form of DAF in combination with an antisense primer 5′GTTGAAAAGGTGGAGACTGG3′, numbered 1269–1288 in the published TM–DAF sequence. PCR was carried out using 2.5 units *Taq* DNA polymerase with the buffers supplied, 1 μl cDNA, 200 nM dNTPs and 10 pmol of each primer in a total volume of 25 μl. Amplification was carried out in a Hybaid Omnigene thermocycler (30 cycles: 94 °C for 30 s, 60 °C for 45 s, 72 °C for 2 min) with a final extension at 72 °C for 15 min. Products were separated on a 1 % agarose gel, transferred to a Hybond N nylon membrane by capillary action and UV-crosslinked. A probe covering an approx. 400-bp-long stretch of sequence common to both forms of mouse DAF (encoding SCR4 and the STP region) was generated by PCR from a plasmid containing the sequence for mouse DAF, with incorporation of dUTP–FITC to give a FITC-labelled probe. The blot was incubated overnight in hybridization buffer [5 × SSPE (1 × SSPE = 10 mM NaH_2PO_4/1 mM EDTA/10 mM NaCl, pH 7.4), 0.5 % (w/v) SDS, 5 × Denhardt's solution, 0.1 mg/ml salmon sperm DNA (denatured at 100 °C for 10 min)] at 55 °C with purified probe, washed twice for 15 min with 2 × SSC containing 0.1 % (w/v) SDS at room temperature, and then twice for 15 min with 0.5 × SSC containing 0.1 % (w/v) SDS at 55 °C. The blot was then washed repeatedly with PBS/0.5 % (v/v) Tween 20, blocked

in PBS containing 5 % (w/v) dried fat-free milk and then probed with polyclonal rabbit anti-FITC followed by goat anti-rabbit HRP. Blots were developed using enhanced chemiluminescence (ECL) (Pierce and Warriner, Chester, U.K.).

Identification and sequencing of alternatively spliced products

Amplification products were ligated into pGEM-T and electrocompetent DH5α bacteria were transformed with the plasmid. Bacterial colonies were chosen and plasmid inserts screened by PCR using primers specific for the SP6- and T7-promoter regions. PCR products were analysed on a 1 % (w/v) agarose gel and reactions that contained an appropriate insert were identified and sequenced on an automated sequencer (ABI PRISM 377 DNA Sequencer, Perkin–Elmer, Warrington, U.K.). Predicted amino acid sequences were analysed for cellular sorting or localization signals using PSORT software (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences; http://psort.nibb.ac.jp/).

Cloning of mouse DAF into expression vectors

DNA encoding mouse DAF was amplified by PCR from mouse testis RNA and reverse transcribed as described above. DNA encoding the TM form of mouse DAF was amplified using the sense primer 5′CCTCAAAACAGCTCCGGCCA3′ and the antisense primer 5′GTTGAAAAGGTGGAGACTGG3′, numbered 1–20 and 1269–1288 respectively in the published TM–DAF sequence [17]. The PCR product was purified, ligated into pCR3.1 and subcloned into the vector pDR2ΔEF1α using restriction enzymes *Nhe*1 and *Eco*RV. DNA encoding the GPI-anchored form of mouse DAF was amplified using the sense primer 5′GGCTCTAGACTTCTACCTGGGGCTATGAT3′ (1–20 in the published GPI sequence, and incorporating a 5′*Xba*I site) and 5′GCGGGATCCTATTGTATCCATTCTTCTTG3′ (antisense, 1254–1273 in the published GPI sequence and incorporating a 5′*Bam*HI site). The PCR product was purified, digested with *Xba*1 and *Bam*H1 and ligated directly into pDR2ΔEF1α expression vector previously excised with the same restriction enzymes. Sequencing confirmed the fidelity of both forms of mouse DAF in the expression vector.

In order to generate a soluble, recombinant form of mouse DAF, DNA encoding the first four SCRs was amplified by RT-PCR from mouse testis RNA and ligated into the expression vector Signal pIgplus, ensuring that it was in frame with DNA encoding the hinge and Fc region of human IgG_1. In order to obtain high levels of secretion, DNA encoding the DAF–Fc fusion protein was subcloned into the high expression vector pDR2ΔEF1α. Sequencing confirmed that no errors had been introduced by PCR.

Transfection of CHO cells

CHO cells, obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.), were seeded into 25-cm^2 flasks. Cells at about 50 % confluency were washed with serum-free medium and transfected with plasmid (4 μg) using Lipofectamine (Life Technologies). Cells were selected using hygromycin B to generate stable lines expressing DAF. The antibiotic was omitted from the medium 24 h before functional assays.

Preparation of cell lysates and Western-blot analysis

Cell lysates were prepared by solubilizing 2×10^7 cells in 1 ml PBS containing 2 % (v/v) Nonidet P40, 10 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM PMSF. Cells were solubilized for 30 min on ice and insoluble debris was removed by centrifugation at 11000 ***g***. Testis tissue lysate was similarly prepared except that one whole testis was homogenized in PBS/10 mM EDTA before solubilization in 500 μl solubilization buffer. A portion of the supernatant was mixed with an equal volume of non-reducing sample-loading buffer, boiled for 2 min and subjected to SDS/PAGE (10 % gel) [25]. Gels were Western blotted on to nitrocellulose, which was subsequently blocked with PBS containing 5 % (w/v) dried-milk powder and probed with polyclonal anti-mouse DAF antiserum (1:1000 dilution). Blots were washed with PBS/0.1 % (v/v) Tween 20 and probed with rabbit anti-rat IgG–HRP (1:1000). After washing with PBS/0.1 % (v/v) Tween 20 and then with PBS alone, blots were developed using ECL.

C3 deposition assay

CHO cells were seeded into 24-well plates at 10^5 cells/well. At 24 h the adherent cells were washed once with complement fixation diluent (CFD) (2.8 mM barbituric acid/145.5 mM NaCl/0.8 mM $MgCl_2$/0.3 mM $CaCl_2$/0.9 mM sodium barbital, pH 7.2; Oxoid Ltd., Basingstoke, U.K.) containing 1 % (w/v) BSA and then incubated with 250 μl of a dilution of rat or mouse immune serum (1:1 mixtures of normal serum and serum from animals immunized with CHO cells). Following a 20 min incubation at 37 °C, serum was removed and 500 μl FACS buffer [PBS/1 % (w/v) BSA/0.05 % (w/v) NaN_3] containing 10 mM EDTA was added to each well to inhibit complement and to detach cells from the plastic. Detached cells were washed in FACS buffer, incubated for 40 min at 4 °C with a 1:400 dilution of rabbit anti-mouse C3, washed and incubated with R-phycoerythrin-conjugated goat anti-rabbit IgG (1:100 dilution). Cells were washed twice, fixed in 1 % formaldehyde and analysed for fluorescence (FACScalibur; Becton Dickinson, Oxford, U.K.). All cells were analysed on identical machine settings.

Cell-lysis assay

CHO cells were seeded into 24-well plates at 10^5 cells/well. At 24 h the adherent cells were washed once with RPMI/5 % (v/v) fetal-calf serum and then incubated for 30 min at 37 °C with 250 μl RPMI/5 % (v/v) fetal-calf serum containing 2 μg/ml calcein acetoxymethyl ester (Molecular Probes, Leiden, The Netherlands). Cells containing calcein were washed with CFD containing 1 % (w/v) BSA before incubation with a dilution of rat immune serum for 30 min at 37 °C. The supernatant was removed and calcein remaining in the cells was released by incubation with 250 μl CFD/1 % (w/v) BSA/0.1 % (v/v) Triton X100 for 15 min. Complement-mediated and detergent-mediated calcein release were measured using a Wellfluor fluorimeter. The percentage lysis for each well was calculated as follows: 100 × complement-mediated release/(complement-mediated release + detergent-mediated release). Background release of calcein from cells incubated in CFD/1 % BSA was also measured.

Purification of DAF–Fc fusion protein and complement inhibition assay

Tissue culture supernatant collected from CHO cells transfected with the vector pDR2ΔEF1α containing an insert encoding the DAF–Fc fusion protein was subjected to affinity chromatography on a Prosep A column. The purified fusion protein was tested in a classical pathway assay. Sheep erythrocytes [2 % (v/v) in PBS] were incubated with Amboceptor (1:500 final dilution) for 15 min

at 37 °C to sensitize the erythrocytes. Sensitized erythrocytes were washed twice in CFD containing 0.1 % (w/v) gelatin and resuspended at a concentration of 2 % (v/v). A sample (50 μl) was added to 100 μl CFD/0.1 % (w/v) gelatin containing a dilution of the complement inhibitor under test and rat serum. The amount of rat serum used had been determined previously to give 40 % lysis in the absence of a complement inhibitor. The cells were incubated for 30 min at 37 °C, pelleted by centrifugation and the A_{415} of the supernatant was measured. For 0 % (background) and 100 % lysis controls, sensitized erythrocytes were incubated with 100 μl CFD containing 0.1 % (w/v) gelatin or 100 μl of water respectively. Lysis in the absence of an inhibitor was also measured. Percentage lysis for each sample was calculated as follows: $100 \times (A_{415}$ sample $- A_{415}$ 0 % control)/(A_{415} 100 % control $- A_{415}$ 0 % control).

RESULTS

Tissue expression of alternative forms of mouse DAF

Northern-blot analysis (Figure 1) and RT-PCR (Figure 2) were used to analyse the expression of DAF in different tissues. DAF mRNA was present in testis, spleen, kidney and liver. In common with other reports, testis and spleen contained particularly high levels of DAF mRNA. A major species at 1.5 kbp was demonstrated together with a second species at 3.5 kbp. As the probe was not designed to distinguish mRNA corresponding to the GPI-linked or TM forms of DAF, RT-PCR was used for this purpose (Figure 2a). The 3′ end of the cDNA was amplified using a sense primer specific for SCR4 and anti-sense primers specific for either GPI-anchored or TM DAF. Amplification of cDNA encoding GPI-anchored DAF resulted in products which were of the predicted size for this form of the protein in all tissues (562 bp; Figure 2a, lane 1). However, amplification using primers specific for the TM cDNA generated products only from testis and spleen RNA; in addition to the predicted 548 bp product, several other amplification products were generated (Figure 2a, lane 2). Southern blotting confirmed that all tissues analysed expressed high levels of the GPI-linked form of DAF, and that testis also strongly expressed the TM form (Figure 2b). Whereas the other tissues tested expressed predominantly the GPI-linked form of DAF, low level expression of TM–DAF was shown by the presence of products of the correct size in kidney, spleen and liver (the last was evident only on over-exposure of the blot to the film). Other RT-PCR products amplified from testis RNA using the TM primer set also hybridized with the mouse DAF probe, indicating that these also comprised the mouse DAF sequence and most likely represented alternative splicing of the gene. The other amplification products in spleen did not hybridize with the probe and were presumably the result of non-specific binding of primers during the PCR reaction. RT-PCR from RNA of each tissue using primers specific for GAPDH and analysis as above demonstrated a specific product of comparable intensity in every tissue (results not shown).

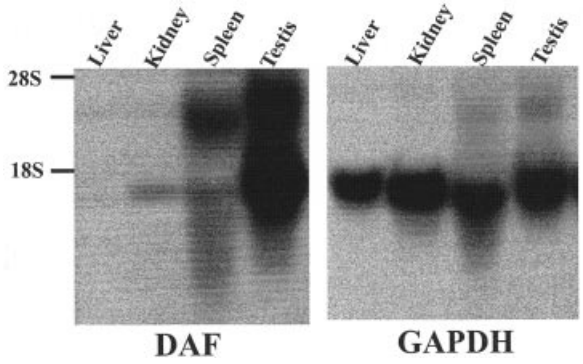

Figure 1 Northern-blot analysis of mouse tissue RNA

Total RNA was isolated from mouse liver, kidney, spleen and testis and electrophoresed on an agarose gel. The RNA was transferred to a nylon membrane and blots were hybridized with probes specific for mouse DAF or GAPDH.

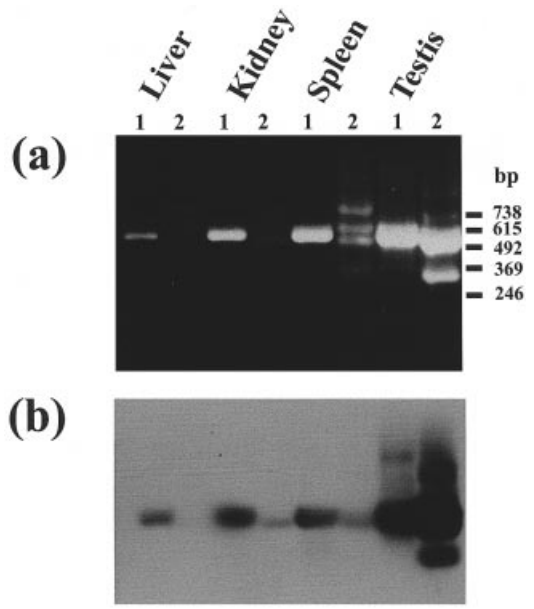

Figure 2 RT-PCR analysis of mouse tissue RNA

(**a**) Total RNA was isolated from liver, kidney, spleen and testis and cDNA was prepared by reverse transcription using oligo(dT). Primers specific for either the GPI-linked form of mouse DAF (lane 1) or TM form of mouse DAF (lane 2) were used in the PCR reaction for each tissue. PCR products were analysed on a 1 % agarose gel. (**b**) The gel shown in (**a**) was blotted on to a nylon membrane and probed with an FITC-labelled probe specific for mouse DAF. Bound probe was detected using rabbit anti-FITC and goat anti-rabbit HRP. Blots were developed using ECL.

Sequencing of alternative forms of mouse DAF

In order to analyse the various mRNA species, RT-PCR from testis and spleen total RNA was carried out using the two primer combinations described above or substituting oligo(dT) as the antisense primer. These two tissues were chosen as they had been shown previously to express TM DAF [17], and our results indicated that testis might have multiple alternative forms of DAF (Figure 2). PCR products were ligated into pGEM-T and inserts were sequenced. This strategy enabled amplification and sequencing of both TM and GPI-anchored products, and also any products with a novel 3′ end. Sequence analysis revealed clones of mouse DAF corresponding to those described previously and also several novel forms. The sequence of SCR4 conformed to the coding sequence for either the *Daf-GPI* gene or the *Daf-TM* gene [17]. However, alternative splicing occurred at the start of the STP region, at points within the STP region, and at the start of the sequence encoding either the TM domain or GPI anchor. In every case splicing occurred at exon/intron boundaries predicted from analysis of human *DAF* gene structure (Figure 3a) [26].

Figure 3 Isoforms of mouse DAF generated by alternative splicing

(**a**) Amino-acid sequences of alternative forms of mouse DAF. The two uppermost sequences are those reported previously as being derived from the *Daf-TM* or *Daf-GPI* gene [17]. Amino acids in the SCR and STP regions that differ between these two forms are indicated by shadowing. Clones 1 and 2 contain the SCR and STP sequence, and clone 3 contains the SCR sequence, described previously as derived from the *Daf-TM* gene. Clone 4 contains the SCR sequence described previously as being derived from the *Daf-GPI* gene [17]. Regions encoding STP-A, -B and -C and the C-terminal domain are indicated; these boundaries were predicted from comparison with the human *Daf* gene structure. Clones 1 and 2 contained the novel GPI-anchoring signal, the altered amino acid is doubly underlined. Broken lines have been introduced to enable line-up of sequences. (**b**) Nucleotide sequence of the GPI-anchor-addition signal and 3′-untranslated region encoded on the *Daf-GPI* gene (top line) and comparison with that found on the alternative gene. Differences in the two sequences are indicated by shadowing and the base difference that results in an altered amino acid is doubly underlined. Sequences resulting from the primer used for PCR are shown in italics. (**c**) Comparison of signal peptides on the two *Daf* genes. The previously published signal peptide on the *Daf-TM* gene [17] is uppermost; the amended form of this signal peptide is compared with that on the *Daf-GPI* gene.

Clone 1, which was isolated from testis mRNA, contained a nucleotide sequence encoding SCR4 and the STP region, which was identical with that encoded by the *Daf-TM* gene. However, at the predicted site for the end of the STP region, the sequence diverged from that of the TM form and was thereafter comparable with the sequence encoding the 3′ end of the GPI form of mouse DAF. The predicted amino acid sequence of the GPI-anchor-addition signal was identical with the published sequence in the *Daf-GPI* gene except for a single amino acid (Val → Ala) (Figure 3a), the result of a single base change in the coding sequence (Figure 3b). There were also several nucleotide differences together with a small deletion (4 bases) in the 3′ untranslated region of the ‘new’ GPI-anchor sequence. Multiple clones of GPI-anchored DAF were sequenced from separate PCR reactions, and in all cases the novel GPI-anchoring signal and 3′-untranslated region were found in clones in which the SCR and STP regions were encoded by the *Daf-TM* gene (four clones). In cases where the SCR and STP regions were encoded on the *Daf-GPI* gene, the nucleotide sequence of the GPI-anchoring signal and 3′-untranslated region were invariably identical with that published previously (a further four clones). Clone 2 (also from testis) was identical with clone 1, except that approximately one-third of the STP region, corresponding to the exon encoding STP-B in human DAF, had been deleted (Figure 3a). Clone 3 had SCRs and a TM domain identical with that reported previously on the *Daf-TM* gene, however the whole STP region had been deleted. This clone was isolated from two separate RT-PCRs of testis RNA, and also from RT-PCR of spleen RNA. In this case the splice sites corresponded to the end of the exon encoding SCR4 and the start of the exon encoding the C-terminal tail. This isoform of DAF may be represented by the smaller RT-PCR product (330 bp) evident in Figure 2(a) (testis, lane 2); this second major product was consistently present following RT-PCR from testis RNA. Clone 4, isolated

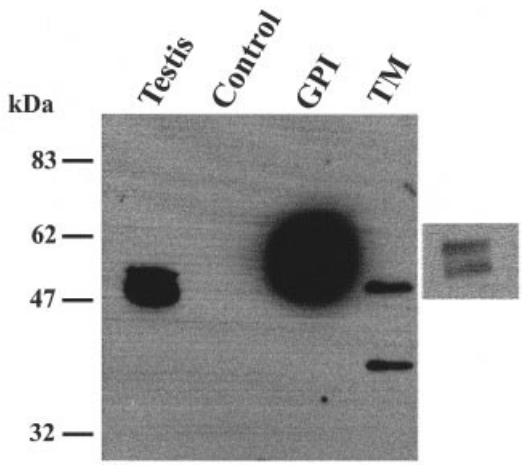

Figure 4 Western blot of mouse DAF-transfected cell lysates and mouse testis tissue lysate

Cultured cells or homogenized testis tissue were solubilized in 2% (v/v) Nonidet P40, and 10 μl of lysate was analysed by SDS/PAGE and Western blot. The blot was probed with polyclonal rat anti-mouse DAF antiserum followed by rabbit anti-rat IgG–HRP. Blots were developed using ECL. Lane 1 (numbering from the left), testis tissue; lane 2, vector control cells; lane 3, cells expressing GPI-anchored mouse DAF; lane 4, cells expressing TM mouse DAF.

from spleen, contained an SCR sequence identical with that published for the *Daf-GPI* gene. The identity stopped at the end of SCR4; there was no STP sequence and no sequence comparable with either the TM domain or GPI-anchor signal. Instead, this clone had a unique sequence following SCR4 which encoded 21 amino acids and the 3′-untranslated region (Figure 3a). Analysis of the sequence using PSORT (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences) software predicted that the protein was likely to be secreted.

Sequencing of the mouse DAF signal peptide

Whilst sequencing plasmids containing full-length clones of TM mouse DAF, we noted a difference in our sequence compared with that shown in the original publication [17]. An insertion of G within the signal peptide (between base number 120 and 121 of the published sequence) altered the reading frame and changed the predicted amino acid sequence of the signal peptide, but not of the mature protein. A new start codon was located which was preceded by the consensus Kozak sequence. When the signal peptide identified in the present work for the TM form of DAF is compared with the published signal peptide of the GPI form, it is evident that they show high levels of similarity (Figure 3c).

Expression of mouse DAF on CHO cells

In order to analyse the function of mouse DAF, full-length cDNAs encoding both TM and GPI-anchored DAF were obtained by RT-PCR from mouse testis RNA. These were cloned into the expression vector pDR2ΔEF1α and sequenced. Several amino acid changes were noted compared with those published previously [17]. There was one amino acid difference in the GPI-anchored form (Glu-101 → Lys; numbering for the mature protein), and three differences in the TM form (Thr-173 → Ile, Ala-176 → Thr and Asp-199 → Asn; all numbering as for the mature protein). These differences were shown not to be PCR artefacts by sequencing several clones from different PCR reactions. In the case of the GPI-anchored clone, the amino acid difference was also present in other published sequences [18,19]. These amino acid changes are likely to represent natural polymorphisms or errors in the original sequence. CHO cells were transfected with the GPI-anchored form, TM form, or vector containing no insert (vector control). Stable lines were generated by selection with hygromycin B and DAF expression was analysed by Western blot and flow cytometry. Analysis by flow cytometry indicated that both were expressed on the membrane but the level of expression of the GPI-anchored form was four times that of TM DAF (results not shown). Treatment of cells with phosphatidylinositol-specific phospholipase C and subsequent analysis by flow cytometry using monoclonal rat anti-mouse DAF antibody confirmed that DAF encoded on the *Daf-GPI* gene could be released (75% release), and that the form encoded by the *Daf-TM* gene was unaffected by this treatment (results not shown).

High level expression of the GPI-anchored form was also evident by Western-blot analysis where it was present as a broad band of apparent molecular mass between 55 kDa and 60 kDa (Figure 4). On short exposures of the blot (inset) a doublet was clearly visible within this broad band. On long exposure of the blot, a band at 38 kDa was also found. The predicted molecular mass of the non-glycosylated protein was 39 kDa, making it possible that the 38 kDa band represents the precursor. The TM form of DAF was evident as sharp bands of molecular mass 40 kDa and 50 kDa. The predicted molecular mass of the non-glycosylated TM protein was 40 kDa. The lower band (40 kDa) is likely to represent the non-glycosylated precursor form of TM–DAF. Attempts to deglycosylate these proteins to confirm the relationship between the different forms have been unsuccessful because of the lability of these proteins in cell lysates under the conditions required for deglycosylation. In mouse

Table 1 C3b deposition on vector control cells and cells expressing the GPI-anchored or the TM form of DAF

CHO cells were incubated in the presence of mouse or rat serum, stained with anti-mouse/rat C3 antibody and analysed by flow cytometry for deposition of C3 fragments on the cell surface. Values shown are the means ± S.D. of three determinations. Statistical significance was analysed by one way analysis of variance and post-hoc analysis by Fisher's least significant difference test with Bonferroni's correction for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

	Median fluorescence			
	5% mouse serum	10% mouse serum	5% rat serum	10% rat serum
Vector control	806 ± 41	1636 ± 73	707 ± 90	1203 ± 130
GPI–Daf	285 ± 29**	380 ± 39***	165 ± 5**	280 ± 12***
TM–Daf	660 ± 70 (ns)	1155 ± 60**	530 ± 32 (ns)	887 ± 39*

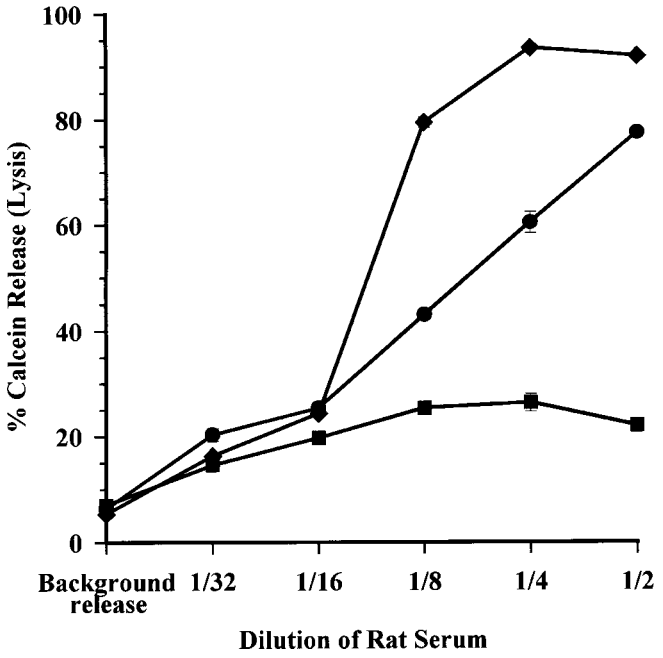

Figure 5 Lysis of CHO cells by rat serum

Cells expressing the GPI-anchored form of DAF (■), the TM form of DAF (●) or vector control cells (◆) were preloaded with calcein and incubated with various dilutions of rat serum. Calcein release and background release (no serum) was measured by fluorimetry. Values shown are the means ± S.D. of three determinations.

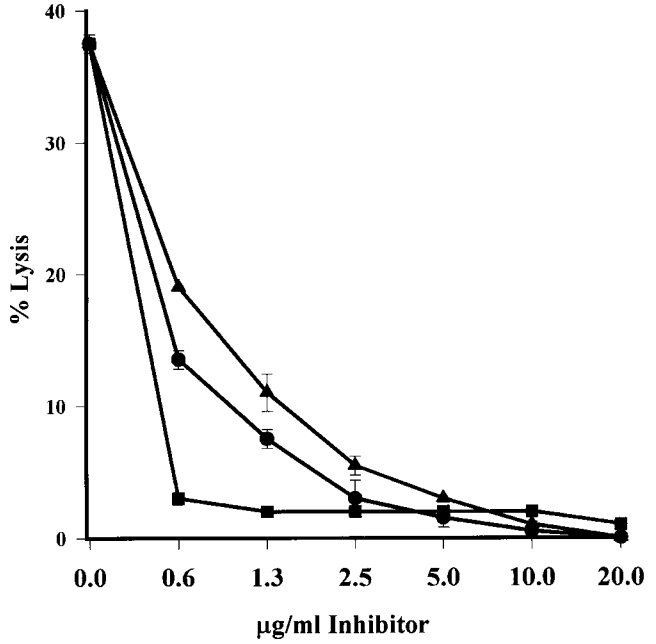

Figure 6 Inhibition of complement by a soluble, engineered form of mouse DAF

Antibody-coated sheep erythrocytes were incubated with rat serum and with dilutions of either mouse DAF–Fc (▲), rat DAF–Fc (●) or soluble recombinant complement receptor 1 (■). Lysis was calculated by measuring haemoglobin release. Values shown are the means ± S.D. of two determinations.

testis lysate, DAF ran as a strong doublet with an apparent mass of 50 kDa and 55 kDa (Figure 4). No reactivity was obtained with vector control cells or blots probed with non-immune rat serum.

Functional analysis of mouse DAF

CHO cells expressing either TM or GPI-anchored mouse DAF were protected from damage by mouse and rat complement. Levels of C3b deposition were markedly reduced compared with control cells exposed to the same serum dilution (Table 1). GPI-anchored DAF caused the greatest reduction in C3 deposition but expression of this form was approximately four-fold higher than that of TM–DAF, making direct comparisons of activities impossible.

Mouse immune serum did not cause lysis of CHO cells, even at high concentrations. As C3b deposition experiments indicated that mouse DAF protected cells equally well from mouse and rat complement attack, rat complement was used to analyse DAF-mediated protection from lysis. Control cells were efficiently lysed by rat serum, whereas cells expressing GPI-anchored DAF were resistant to cell lysis at the serum dilutions used (Figure 5). Expression of the TM form of DAF conferred an intermediate level of protection.

In order to assess whether a soluble form of mouse DAF, which lacked an STP region, could regulate complement from the fluid phase, we constructed a recombinant molecule which consisted of the four SCR domains of GPI–DAF linked to the Fc region of human IgG_1. This strategy yielded an 'antibody-like' molecule which was easy to purify by Protein A affinity chromatography. The mouse DAF–Fc fusion protein efficiently inhibited lysis of antibody-coated sheep erythrocytes by rat complement, indicating that a soluble form of Daf lacking the STP could function as a complement regulator (Figure 6).

DISCUSSION

The original cloning of mouse DAF identified two cDNAs encoding respectively TM and GPI-anchored forms of the protein, derived from separate but highly homologous genes (*Daf-TM* and *Daf-GPI*) [17]. Based upon studies of mRNA expression, GPI-DAF was reported to be widely distributed, whereas TM–DAF was abundant only in testis and expressed at low level in a few other tissues. In the present work, we confirm that both GPI-anchored and TM forms of mouse DAF are abundant in testis; in other tissues the GPI-anchored form predominated but, using sensitive detection techniques, low level expression of the TM form was found in all tissues tested (Figure 2). In addition to mRNAs of the anticipated sizes, we detected several other DAF-related mRNAs, suggestive of novel forms of mouse DAF. We have characterized several of these mRNAs and in the present study demonstrate that alternative splicing, analogous to that reported for DAF in other species, occurs in both mouse *Daf* genes. Alternative splicing of the *Daf-TM* gene generated both TM and GPI-anchored forms of DAF. Two different GPI-anchoring signals were demonstrated by RT-PCR from Balb/C mouse testis RNA (Figure 3b). The first corresponded to that described previously for the *Daf-GPI* gene, and was consistently found on isoforms in which the SCR and STP sequence was also derived from this gene. The second signal was always found on isoforms in which the SCR and STP sequence was derived from the *Daf-TM* gene. Several different clones encoding each of the above isoforms were isolated and characterized, making it most unlikely that either was derived from a

cloning artefact. It is interesting to note that in the recently described *Daf-GPI* knockout mouse, mRNA specific for GPI-anchored DAF was not detected in lung or intestine by Northern-blot analysis, confirming the phenotype; however, a faint signal was detected in testis [26a]. Expression of the *Daf-TM* gene product was unaffected in this knockout mouse. The demonstration in the present work that the *Daf-TM* gene can yield GPI–DAF provides a clear explanation for this unexpected finding in the knockout, which is further supported by the observation that the size of the mRNA bound by the GPI-specific probe in the knockout was slightly different in size from the predominant signal in normal mice (W. C. Song, personal communication).

Two splice variants of the *Daf-TM* gene were identified which contained the novel GPI-anchoring signal sequence. One of these variants (clone 1) had a full-length STP region (77 amino acids), whereas the other (clone 2) had a truncated STP region (56 residues). Comparison of the mouse and human DAF sequences and prediction of the exon boundaries in the mouse *Daf* genes demonstrated that the missing region corresponded exactly to the region encoding STP-B. A third new isoform derived from the *Daf-TM* gene had no STP region and was the result of splicing out of the exons predicted to encode STP-A, -B and -C; this isoform retained the TM anchoring sequence (clone 3). A splice variant derived from the *Daf-GPI* gene was found, which is predicted to encode a secreted form of the molecule, consisting of the four SCRs and a short C-terminal domain only (clone 4).

There are numerous precedents for alternative splicing generating multiple isoforms of DAF and the related complement regulator MCP. In guinea pig, TM, GPI and secreted forms of DAF are all generated from a single gene by exon splicing which introduces a frameshift, resulting in alteration of the amino acid sequence of the C-terminal region of the mature protein [20]. There are four contiguous STP-encoding regions in guinea pig DAF; differential usage of splice sites in one exon results in insertion or deletion of the second and third STP regions. The fourth STP region (S/T-d) is encoded on two exons and is present in all forms of guinea pig DAF so far identified. Rat DAF also exists in at least two different forms generated by splicing of a single gene; GPI-anchored DAF and a second form which appears to be secreted [22]. In humans, an alternatively spliced form of DAF has been identified which encodes a protein differing in its C-terminal region, which is predicted to be a secreted molecule, although it has yet to be characterized in plasma [3]. The human *Mcp* gene exhibits complex alternative splicing, producing isoforms differing in the length of the STP region and expressing one of two different cytoplasmic tails [5]. Common forms of human MCP have either one or two STP regions, although several other isoforms have been demonstrated by RT-PCR, some of which are tissue specific in their expression.

Although mouse DAF was first cloned several years ago, no functional analysis has been reported. In order to confirm that mouse DAF has the capacity to inhibit complement, the major forms (TM and GPI-anchored) were expressed at high levels in CHO cells. Expression was confirmed using newly derived monoclonal and polyclonal antibodies [24]. Expression of either TM–DAF or GPI–DAF inhibited deposition of C3 on CHO cells exposed to rat or mouse immune serum and protected the cells from classical pathway-mediated lysis using rat serum as a source of complement (Table 1 and Figure 5). The other isoforms of mouse DAF that we describe here have yet to be tested for function. It will be of interest to examine whether the different isoforms of DAF preferentially inhibit a particular activation pathway. It has been shown previously that the C3b/C4b binding SCRs in human complement receptor 1 have specificity for only one of these ligands, and that alteration of just a few amino acids can change specificity from C3b to C4b [27,28]. Several of the novel isoforms of DAF described here are membrane-bound molecules with truncated (clone 2) or absent (clone 3) STP regions. In light of the report that an engineered form of human DAF lacking the STP region was incapable of regulating complement [29], it will be of interest to examine the capacity of these mouse DAF isoforms to inhibit complement. In the case of the human molecule, loss of complement regulatory capacity was shown to be a consequence of the distance of the functional domains from the plasma membrane, as replacement of the STP region with a spacer derived from HLA B44 restored function. Functional analysis of guinea pig DAF isoforms indicated that those with long STP regions inhibited complement more efficiently [21]. Total deletion of the STP region of DAF, as shown in clone 3, has not been demonstrated in other species. However, total STP deletion has been demonstrated in human, rat and guinea pig MCP [30–32]. The ability of these forms to bind C3b and regulate complement was not tested.

The DAF clone 4, consisting only of SCR domains and a short, unique C-terminus, with no STP region, is predicted to encode a secreted form of mouse DAF. We do not yet have any evidence that this form is present in plasma in the mouse or that it has any function. Recombinant soluble forms of human DAF, consisting of the four SCRs and STP region, have been generated and shown to retain decay accelerating activity, thus indicating that DAF can function in the fluid phase [33,34]. In the present study, we show that a soluble, recombinant form of mouse DAF, consisting of only the four SCR domains, linked to the Fc region of human IgG_1, regulates rat complement, indicating that soluble mouse DAF might be functional *in vivo* and that the STP region is not necessary for regulation from the fluid phase.

The different isoforms of mouse DAF might also have specific roles other than complement regulation. Human GPI-anchored DAF has the capacity to signal, whereas a TM form generated using recombinant techniques lacked signalling ability [35,36], indicating that the TM and GPI-linked isoforms of mouse DAF will have different signalling capacities. Some isoforms of mouse DAF may act as receptors for C3 metabolites or even as cellular receptors for various viruses. In humans, DAF is a receptor for enterovirus, echovirus and coxsackie virus [37–39] and the related molecule, MCP, is a receptor for measles virus [40]. Isoforms of human MCP, differing in the length of STP region, vary in the efficiency with which they mediate measles virus infection [41]. Different isoforms of mouse DAF may exhibit specific interactions with viruses or other micro-organisms and vary in their ability to mediate infection. Of particular note is the high level of expression of multiple isoforms of mouse DAF in the testis (Figure 1, Figure 2 and Figure 4). The abundance of expression at this site provokes the suggestion that DAF may play a role in the development of spermatozoa, aid survival of spermatozoa in the female genital tract or fulfil other important roles in reproduction.

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