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Immunology

Cloning and characterization of cDNAs encoding the complete sequence of decay-accelerating factor of human complement

(paroxysmal nocturnal hemoglobinuria)

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ABSTRACT **cDNAs encoding the complement decay-accelerating factor (DAF) were isolated from HeLa and differentiated HL-60 λgt cDNA libraries by screening with a codon preference oligonucleotide corresponding to DAF NH_2-terminal amino acids 3–14. The composite cDNA sequence showed a 347-amino acid protein preceded by an NH_2-terminal leader peptide sequence. The translated sequence beginning at the DAF NH_2 terminus encodes four contiguous ≈61-amino acid long repetitive units of internal homology. The repetitive regions contain four conserved cysteines, one proline, one glycine, one glycine/alanine, four leucines/isoleucines/valines, one serine, three tyrosines/phenylalanines, and one tryptophan and show striking homology to similar regions previously identified in factor B, C2, C4 binding protein, factor H, C1r, factor XIII, interleukin 2 receptor, and serum β_2-glycoprotein I. The consensus repeats are attached to a 70-amino acid long segment rich in serine and threonine (potential O-glycosylation sites), which is in turn followed by a stretch of hydrophobic amino acids. RNA blot analysis of HeLa and HL-60 RNA revealed three DAF mRNA species of 3.1, 2.7, and 2.0 kilobases. The results indicate that portions of the DAF gene may have evolved from a DNA element common to the above proteins, that DAF cDNA predicts a COOH-terminal anchoring polypeptide, and that distinct species of DAF message are elaborated in cells.**

Avoidance by host tissues of attack by autologous complement proteins is dependent in part on the activities of membrane regulatory factors. One molecule involved in this control is a 70-kDa glycoprotein termed decay-accelerating factor (DAF) (reviewed in ref. 1). This surface component recognizes C4b and C3b fragments that condense with cell-surface hydroxyl or amino groups when nascent C4b and C3b are locally generated during C4 and C3 activation. Interaction of DAF with the cell-associated C4b and C3b polypeptides interferes with their ability to catalyze the conversion of C2 and factor B to enzymatically active C2a and Bb and thereby prevents the formation of C4b2a and C3bBb, the amplification convertases of the complement cascade. Interruption by DAF of the complement sequence at this early step in activation efficiently halts progression of the cascade and prevents consequent cell injury.

In humans, DAF is expressed on the plasma membranes of all cell types that are in intimate contact with plasma complement proteins (2–4). The regulatory factor is also found on the surfaces of epithelial cells lining extracellular compartments, and variants of the molecule are present in body fluids and in extracellular matrix (5). Abrogation of DAF activity in normal erythrocytes (E^{hu}) abolishes the inherent resistance of the erythrocytes to uptake of C3b and increases their susceptibility to lysis (6, 7). Conversely, restoration of DAF activity to affected E^{hu} from patients with paroxysmal nocturnal hemoglobinuria, a disorder in which DAF is deficient (2, 7–9), diminishes the exaggerated C3b uptake (10), which characterizes the complement-sensitive cell populations *in vivo*.

Cell-associated DAF is an amphipathic protein that is anchored in cell membranes by a covalently linked glycolipid (11, 12) rather than by a polypeptide anchor comprised of hydrophobic amino acids. This non-amino acid structure is composed of a phospholipid consisting of fatty acids, glycerol, and inositol (E. I. Walter, W. H. Roberts, T. L. Rosenberry, and M.E.M., unpublished observations) linked to an oligosaccharide containing nonacetylated glucosamine and ethanolamine (11) and is similar to anchoring structures that have been described in trypanosome and leishmania (membrane form) variant surface glycoproteins (mfVSGs) (reviewed in ref. 13), murine thymocyte Thy-1 antigen (reviewed in ref. 14), and E^{hu} acetylcholinesterase (15). In mfVSGs and E^{hu} acetylcholinesterase, the glycolipid is attached via an amide bond to the COOH-terminal amino acid of each protein. Sequence analysis of cDNAs that encode mfVSGs and Thy-1 antigen has indicated that these proteins are initially synthesized with COOH-terminal extension peptides typical of conventional anchors and that these peptides are replaced by the glycolipid anchoring structure during a posttranslational modification.

In this communication, we report the identification of two overlapping DAF cDNAs and their use for determination of DAF protein sequence and analysis of its features.

METHODS

Reagents. Recombinant DNA enzymes were purchased from New England Biolabs and Pharmacia. pGEM1 and pBS plasmids and protoclone λgt10 and λgt11 systems were obtained from Promega Biotec (Madison, WI) and Stratagene Cloning Systems (San Diego, CA). Radionuclides were from New England Nuclear; oligo(dT)-cellulose type 7 and oligo$(dT)_{12-18}$ primer were from Pharmacia. Colony/Plaque-Screen transfer membranes and GeneScreen*Plus* were purchased from New England Nuclear and Schleicher & Schuell (Keene, NH), and nick-translation kits were from Bethesda Research Laboratories.

DAF Protein Analyses. E^{hu} DAF was purified from Nonidet P-40 extracts of stroma by affinity chromatography (11) using Sepharose coupled to (IA10) monoclonal anti-DAF antibody

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Abbreviations: DAF, decay accelerating factor; E^{hu}, human erythrocytes; mfVSG, membrane form variant surface glycoprotein.

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(2). For NH_2-terminal sequencing, 0.1- to 1.0-nmol samples of affinity-purified DAF were electrodialyzed against 0.01 M NH_4HCO_3/0.001% $NaDodSO_4$, pH 8.0, concentrated, and were subjected on Polybrene-coated discs to automated Edman degradation in an Applied Biosystems (Foster City, CA) 470A sequencer with a 120A PTH Analyzer. For internal sequence, 0.5 nmol of reduced and alkylated DAF in 70% trifluoroacetic acid was digested with CNBr (10 mg/ml) for 20 hr at 20°C under N_2. Fragments were separated by reverse-phase HPLC (Applied Biosystems 130A system) using a Vydac C8 column (Alltech, Deerfield, IL) and analyzed as described above. DAF protein levels in cell extracts were quantitated by immunoradiometric assay using IA10 as capturing reagent and anti-DAF monoclonal antibody IIH6 (directed against a different DAF epitope) as revealing agent (2).

Oligonucleotide Probes and Screening of cDNA Libraries. The oligonucleotide probe was synthesized in an Applied Biosystems 380A DNA synthesizer by the phosphoramidite method (16). Failure sequences were eliminated by PAGE, and after end-labeling with [γ-^{32}P]ATP (4×10^4 cpm/pmol), unincorporated label was removed by gel filtration.

Total cellular RNA was isolated by the guanidine isothiocyanate/CsCl method (17) from HeLa cells (10^9) grown in monolayers or from HL-60 cells (2×10^9) grown in suspension for 72 hr in 1% dimethyl sulfoxide. Poly(A) RNA was purified by oligo(dT)-cellulose chromatography. cDNA libraries were prepared from 5 μg of HeLa or HL-60 poly(A) RNA by the method of Gubler and Hoffman (18). HeLa cDNA inserts [>0.5 kilobase (kb)] were ligated into λgt10 arms and HL-60 inserts (>1.5 kb) were ligated into λgt11 arms. Additional size-selected (>1 kb) λgt10 and λgt11 HeLa cDNA libraries in which second-strand synthesis was performed conventionally using the Klenow fragment of DNA polymerase I were provided by T. Nilson (Case Western Reserve University). After packaging, the Gubler and Hoffman λgt10 and λgt11 libraries were screened in C600hfl and Y1090 *Escherichia coli*, and the conventional HeLa libraries in C600 and Y1088 *E. coli*, respectively.

Duplicate plaque lifts on nitrocellulose or Colony/Plaque-Screen transfers were hybridized (19) overnight at 42°C in 20% formamide, 6× SSC (1× SSC = 0.15 M sodium chloride/15 mM sodium citrate), 5% dextran sulfate, 0.1% $NaDodSO_4$, 5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone) containing 0.1 mg of sonicated herring sperm DNA per ml, using labeled probe ($\approx 10^5$ cpm/ml). The hybridized transfers were rinsed in 2× SSC, 0.1% $NaDodSO_4$, washed two times for 30 min with 2× SSC, 0.1% $NaDodSO_4$ at 30°C, and autoradiographs were prepared at −70°C on Kodak X-Omat XAR film. Plaques corresponding to signals that were positive on duplicate transfers were plaque-purified to homogeneity.

Recombinant λ DNA and Subcloning. λgt10 or λgt11 DNA was prepared by standard techniques (20). cDNA inserts were subcloned into the *Eco*RI sites of pGEM1 or pBS in both orientations (20).

Sequence Determinations and Computer Data Analysis. DNA sequencing was performed by the Maxam–Gilbert method (21) using a fifth confirmatory digestion with 1.2 M NaOH at 90°C for 8 min for adenine and cytosine identification. Fragments were end-labeled at 3′ recessive ends using Klenow fragment. Selected fragments of DF1 were also sequenced by dideoxy-chain termination (22). Nucleotide sequence analyses and homology searches were conducted using the DNASIS program and GenBank Genetic Sequence Data Bank.§

§National Institutes of Health (1986) Genetic Sequence Databank: GenBank (Research Systems Div., Bolt, Beranek, and Newman, Cambridge, MA), Tape Release 44.0.

RNA Blot Analyses. RNA blot transfers were prepared from poly(A) RNA (5–10 μg per lane) that was fractionated on 2.2 M formaldehyde/0.9% agarose horizontal slab gels (20) and transferred to nylon by electrotransfer. cDNA probe labeled with [α-^{32}P]dCTP by the random hexanucleotide-primed method (23) was added to the filters, hybridization was performed at 42°C in 6× SSC/50% formamide/0.1% $NaDodSO_4$/1× Denhardt's solution/12.5% dextran sulfate/10 mM Tris·HCl, pH 7.6, the filters were washed at 56°C in 0.2× SSC/0.1% $NaDodSO_4$, and autoradiographs were prepared as described above.

RESULTS

For the preparation of an oligonucleotide probe able to hybridize to DAF cDNA, a positive strand codon-preference 36-mer (GGC-CTG-CCC-CCT-GAT-GTG-CCC-AAT-GCC-CAG-CCT-GCC) was synthesized corresponding to NH_2-terminal amino acids 3–14 (Gly-Leu-Pro-Pro-Asp-Val-Pro-Asn-Ala-Gln-Pro-Ala) determined from Edman sequencing of the DAF NH_2 terminus. This probe was purified on gels and, after end-labeling with ^{32}P, was used to screen four cDNA libraries (see *Methods*). Screening of 1–2 × 10^5 recombinant plaques from each library yielded a total of 14 positive clones, which were designated λDF1-λDF14. HL-60-derived λDF1 and λDF2 containing inserts of ≈1900 and ≈1750 base pairs (bp), respectively, were chosen for analysis.

DF2 was subcloned into pGEM1 and DF1 into pBS. As shown in Fig. 1, all restriction sites in the overlap region of the two cDNAs coincided. The shorter DF1 insert lacked ≈250 bp of sequence present at the 3′ end of DF2 but extended ≈90 bp beyond the 5′ end of DF2. The sequencing strategy for the two cDNAs is diagrammed in Fig. 1. DF2 was sequenced in its entirety and portions of DF1 were then sequenced to identify nucleotides upstream from the DF2 5′ terminus and to determine the relationship between the two inserts. The sequence of DF2 at the 5′ end corresponded to DAF NH_2-terminal amino acids 3–14 used to construct the probe (showing 83% homology with that of the oligonucleotide) and, at the 3′ end, consisted of a poly(A) track (11 adenines) preceded (18 nucleotides upstream) by an AATAAA polyadenylylation signal. The sequence of DF1 coincided precisely with that of DF2 at all shared nucleotide positions identified and the two diverged only at the DF1 3′ end where a poly(A) track (41 adenines) and a possible upstream unconventional polyadenylylation signal contained within the sequence AAATAGA were found that aligned with an identical sequence 277 bp upstream from the 3′ end of DF2. The translated amino acid sequence of the two cDNAs corresponded precisely to all amino acid assignments outside of the probe made by Edman protein sequencing (see Fig. 2).

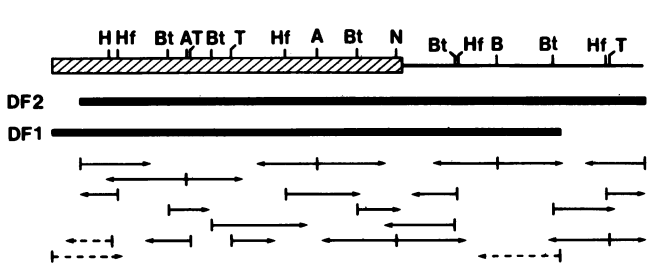

FIG. 1. Sequencing strategy for DAF cDNAs. The coding and 3′ untranslated regions of DAF cDNA are shown schematically by the hatched bar and solid line, respectively, and the DF2 and DF1 inserts are shown by the solid bars. Letters show the relevant cleavage sites of the enzymes *Hin*dIII (H), *Hin*fI (Hf), *Bst*NI (Bt), *Nco* I (N), *Ava* II (A), *Taq* I (T), and *Bgl* II (B) used for sequencing. Arrows arising from endonuclease sites indicate the direction and extent of analysis of the respective fragments. Solid arrows, DF2 fragments; dashed arrows, DF1 fragments.

These included 1 (11), 15–19, and 21–23 obtained from the DAF NH_2 terminus, and 226, 228, 230–232, and 234–239 obtained from a CNBr DAF peptide.

As shown in Fig. 2, the composite DAF cDNA sequence encodes a protein of 347 amino acids preceded by a hydrophobic amino acid-rich stretch characteristic of an NH_2-terminal leader sequence. No initiation codon (24) is apparent within this region, indicating that it is incomplete. The 3′ end of the coding sequence displays hydrophobic amino acids appropriate for COOH-terminal polypeptide anchorage. The overall polypeptide encoded by the translated sequence corresponds in size to the 46-kDa *in vitro* translation product of (HeLa cell) DAF mRNA (M.E.M., W. H. Mann, and M. Rosenfeld, unpublished observations) and intracellular DAF precursors (11, 25) identified in previous studies. The presence of a single potential N-glycosylation site at position 61 and multiple potential O-glycosylation sites in the serine- and threonine-rich region between amino acids 253 and 322 are consistent with previous endoglycosidase analyses of DAF protein, which showed one N-linked oligosaccharide unit and multiple O-linked oligosaccharide units (25).

Within the 347-amino acid translated DAF sequence are four contiguous repeats of internal homology of ≈61 amino acids. These repeats, which start at the NH_2 terminus of the processed protein and are bounded by conserved cysteine residues, are aligned in Fig. 3. The repetitive units encompass amino acids 1–63, 64–125, 126–187, and 188–250 and contain four cysteines, one proline, one glycine, one glycine/alanine, four leucines/isoleucines/valines, one serine, three tyrosines/phenylalanines, and one tryptophan, which are conserved. Overall amino acid homologies between repeats 1 and 2, 2 and 3, 3 and 4, and 2 and 4 are 20%, 24%, 30%, and 18%, respectively. The nucleotide sequences for the conserved residues show extensive codon variability.

To ascertain the nature of mRNA recognized by DAF cDNA and to establish whether levels of mRNA detected in cells by DF2 DNA correlate with DAF protein expression, labeled DF2 insert was used to screen RNA blots prepared with mRNA isolated from HeLa cells, uninduced- and 1,25-dihydroxyvitamin D_3-induced HL-60 cells, and HSB-2 cells, which express 2×10^5, 6 and 14×10^4, and $<10^3$ molecules of DAF per cell, respectively. As shown for HeLa cell mRNA (Fig. 4), in all cell types under conditions of high stringency, DF2 hybridized to two major mRNA species of 2.7 and 2.0 kb and a minor mRNA species of ≈3.1 kb. The levels of the 2.7- and 2.0-kb mRNA species in each cell type correlated with the (above) levels of DAF protein determined by immunoradiometric assay.

DISCUSSION

The two DAF cDNA clones described in the present study were identified by using an oligonucleotide probe corresponding to NH_2-terminal amino acids 3–14. The presence in the cDNAs of derived amino acid sequences corresponding to DAF amino acids 1 (11), 15–23, and 226–239 (with three Edman cycle blanks) determined by protein sequencing as

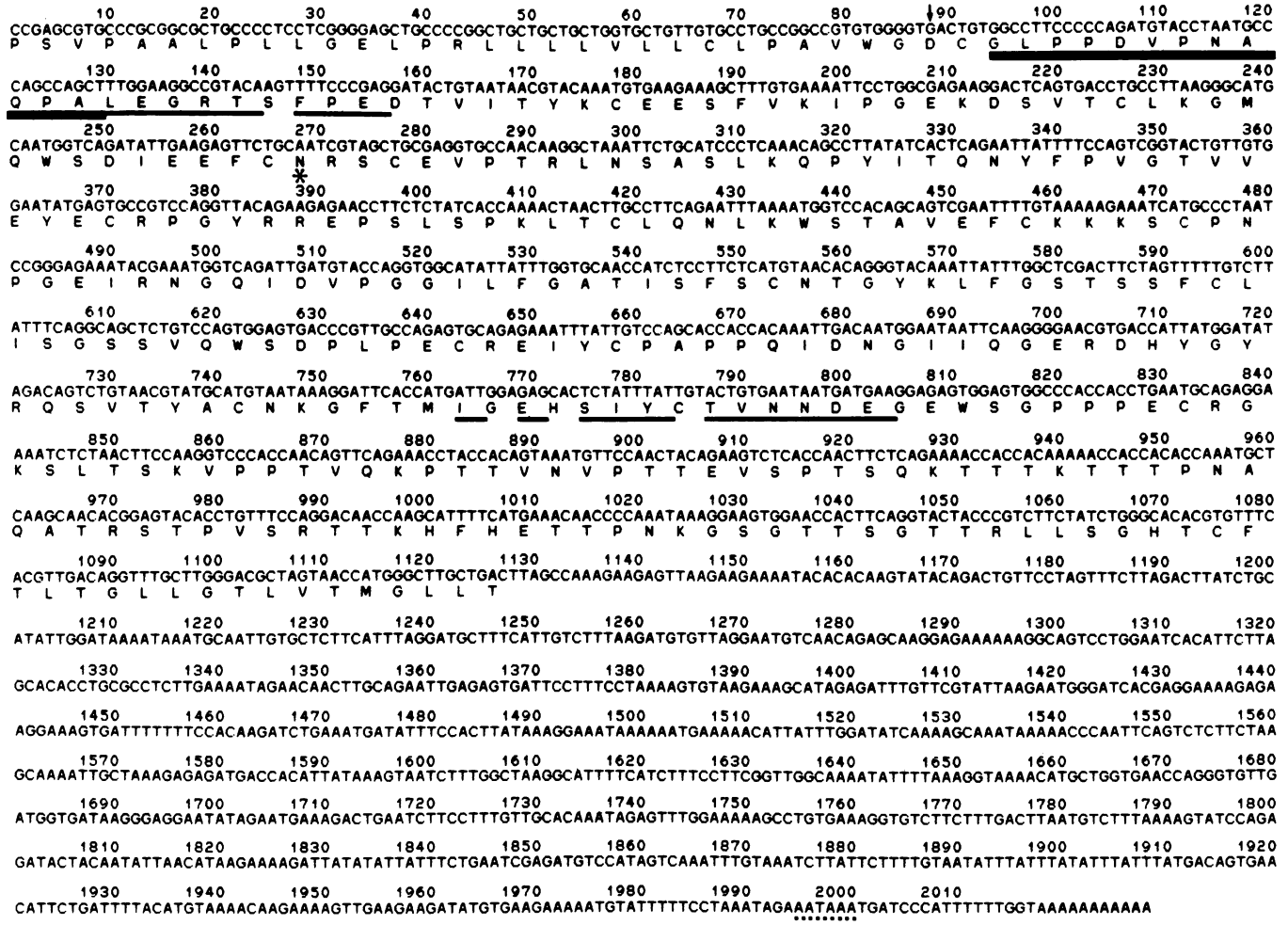

FIG. 2. Composite nucleotide and derived amino acid sequences of DF2 and DF1 cDNAs. The sequence used to construct the probe is heavily underlined and the sequences outside the probe determined by Edman protein sequencing are lightly underlined. Amino acids are identified by the single-letter code. The single potential N-glycosylation site is indicated by an asterisk. The polyadenylylation signal AATAAA is underlined with a dotted line. The protein NH_2 terminus is indicated by the arrow.

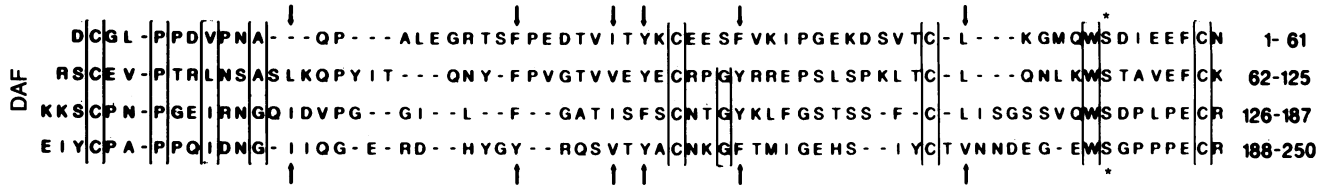

FIG. 3. Alignment of the repetitive sequences encoded by DAF cDNA. Conserved amino acids (identified by the single-letter code) that are present in all four repeats of DAF as well as in the repeats of previously studied proteins (see text) are indicated by open boxes. Additional conserved amino acids present in multiple repeats of DAF (and variably conserved in the other related proteins) are indicated by asterisks and functionally equivalent amino acids defined by (K = R = H), (Q = N), (V = L = I), (S = T), (F = W = Y), and (E = D) are indicated by arrows. The protein positions of the DAF peptides are indicated.

well as to amino acids 3–14 used to construct the probe establish that these cDNAs encode DAF sequence. The DF2 insert, extending from the third nucleotide for DAF NH_2-terminal amino acid 2 through a poly(A) tail, and the DF1 insert, beginning 92 bp upstream from DF2 and hence encompassing the NH_2-terminal signal peptide (and DAF amino acids 1 and 2) collectively showed all the elements of the complete DAF coding sequence except for a translational start site, which could not be identified using these clones. The sequence of the two cDNAs coincided at all overlapping positions except at the 3′ end of DF1, where a poly(A) tract was found preceded by a possible upstream unconventional AAATAGA polyadenylylation signal. The alignment of this subsequence with the same sequence upstream in DF2 suggests that the two cDNAs may have derived from alternatively polyadenylylated mRNAs transcribed from the same DNA element.

A prominent feature of the DAF cDNA is the presence within the coding sequence of four repetitive regions of internal homology. The repetitive polypeptide sequences derived from these regions have an average length of 61 amino acids and are characterized by the presence of conserved sequences of Cys---Pro--Leu/Ile--Ala/Gly at their NH_2-terminal extremity, Cys--Gly and Cys-Leu internally, and Trp------Cys at their COOH-terminal extremity with additional conserved residues interspersed with variable regularity. These repeating sequences show not only extensive homology with each other but also striking homology with repetitive sequences that recently have been described in several other proteins (reviewed in ref. 26). Four such regions of complete and seven of partial homology are contained in 150-kDa human factor H, as shown by analyses of partial cDNAs and as many as 20 predicted by analogy to murine factor H. Eight such units are contained in each of the 70-kDa monomeric subunits of 550-kDa C4 binding protein and there is evidence that similar units are present in the functionally analogous C3b/C4b receptor (CR1). Three repetitive regions are contained in the 25-kDa C2b and 30-kDa Ba fragments of C2 and factor B, respectively. Finally, two such repeats are found in the A chain of C1r, ten in the β subunit of factor XIII, five in the 50-kDa serum protein β_2-glycoprotein I, and two in the 55-kDa membrane protein interleukin 2 receptor. The repeats begin at the NH_2 terminus in all of the proteins except C1r. With a few exceptions, the following residues are conserved in each of the proteins: four cysteines, two glycines, one proline, one leucine/isoleucine, and one tryptophan. The repeats in factor H and C4 binding protein show the greatest homology with those in DAF.

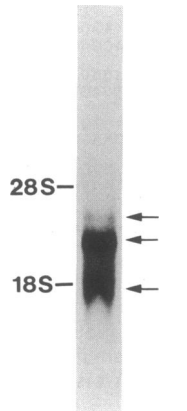

FIG. 4. RNA blot hybridization analysis of HeLa cell mRNA using DF2 as probe. A nitrocellulose transfer of electrophoresed HeLa cell poly(A) RNA (5 μg) was hybridized with ^{32}P-labeled DF2 cDNA and autoradiographs of the washed blots were prepared. The relative migrations of 28S and 18S RNA standards are shown. Three bands corresponding to mRNA species of 2.7, 2.0, and 3.1 kb were identified.

The evolutionary significance of the repetitive sequences in DAF and these other proteins is as yet unclear. Portions of these proteins may have evolved from a common ancestral DNA element. If so, they have become widely dispersed in the genome. While factor B and C2 are closely linked on chromosomes 6 in humans (27) and 17 in mouse (28), factor H, C4 binding protein and CR1 have been shown to be separately linked in a gene cluster (29) on chromosome one in humans (30). Moreover, in humans, the gene for interleukin 2 receptor is present on chromosome 10 (31). The chromosomal localization of DAF is not yet known. The low sequence homology among these repeats at both amino acid and nucleotide levels and the considerable codon variability for the conserved amino acids argues against an active gene conversion mechanism driving conservation of this sequence feature, as is the case for some gene families (32). Furthermore, these observations suggest that the conserved units are not simply evolutionary vestiges and suggest a biological role for this substructural feature (33, 26).

Factor H, C4 binding protein, CR1, factor B, and C2 are all C3b and/or C4b binding proteins as is DAF (1, 26, 33). Each recognizes activation-dependent sites in the structurally homologous C3b and C4b molecules, which deposit in clusters on targets. Factor H, C4 binding protein, CR1, and DAF all interfere with the association of C2 and factor B with C4b and C3b and could interact with a common domain on the clustered C4b and C3b molecules. The higher molecular weight regulatory proteins, factor H, C4 binding protein, and CR1, also function as cofactors for cleavage of the α' chains of C3b and C4b by the serine protease factor I, whereas DAF has no cofactor activity (7). A schematic representation of the structural features of DAF is shown in Fig. 5. It is likely that the repeating units serve as the C3b/C4b binding domains of DAF and these other molecules. While the repeats in C1r could similarly be involved in C4b interaction, the function of the same repetitive units in β_2-glycoprotein I, factor XIII, and interleukin 2 receptor, proteins not known to interact with C3b or C4b, is unclear.

The sequence following the repeats (253–322), which contains numerous serine and threonine residues, potential sites of O-linked glycosylation (11, 25), corresponds to the region of DAF immediately adjacent to the cell membrane. Similar serine- and threonine-rich regions just external to the membrane are sites of clustered O-linked oligosaccharides in the low density lipoprotein receptor (34) and are also found in interleukin 2 receptor (35). The functional significance of this structural feature is unknown.

The hydrophobic amino acid sequence at the COOH terminus of the translated DAF sequence resembles COOH-

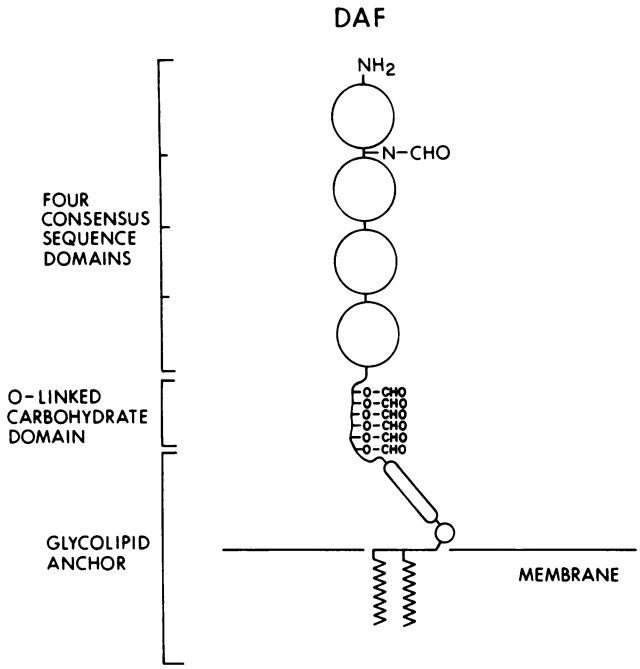

FIG. 5. Model of membrane DAF based on DAF cDNA sequence and protein analyses (11, 25). The four repeating units start at the NH_2 terminus of the DAF molecule. The position of the single N-glycosylation site and probable locations of multiple O-glycosylation sites are indicated. The COOH-terminal polypeptide anchor predicted by the cDNA has been replaced by the glycolipid anchor known to be present in the mature DAF molecule (11).

terminal sequences derived from the cDNAs that encode mfVSGs (13) and Thy-1 antigen (14). The extensions in mfVSGs and Thy-1 that are eliminated prior to addition of the glycolipid anchor are 14–31 amino acids long and are similarly devoid of terminal charged residues present in transmembrane polypeptide anchors. Although precise localization of the corresponding region in DAF must await identification of the DAF COOH-terminal amino acid, hydrophilicity plots show a marked increase in hydrophobicity starting 24 amino acids from the COOH terminus of the molecule. The signal, present within glycolipid anchored proteins, that directs removal of the COOH-terminal extension peptide and substitution of the glycolipid tail during biosynthesis is not known. Studies of mfVSG biosynthesis have shown that anchor substitution takes place within 1 min of appearance of VSG polypeptide on polysomes (13), raising the speculation that the COOH-terminal extension functions as a stop sequence that serves to correctly position the translation product in the endoplasmic reticulum for attachment of the glycolipid anchor. Investigations of DAF biosynthesis in HeLa cells (11) have similarly demonstrated that glycolipid components are incorporated prior to processing of the pro-DAF molecule in the Golgi complex.

Analysis of poly(A) RNA from HeLa, HL-60, and HSB-2 cells on RNA blots using labeled DF2 as probe revealed three mRNA species that correlated quantitatively on blots with levels of DAF protein substantiating that the bands represent authentic DAF mRNA and indicating a transcriptional control of relative DAF expression in these cells. The different mRNA species could represent products of different DAF genes and/or of alternative mRNA splicing. Two of the mRNA species identified (3.1 and 2.7 kb) are larger than the composite sequence of DF2 and DF1, which comprises a full-length DAF coding sequence and presumably complete 3′ untranslated region. Hence, either DF2 and/or DF1 mRNA species correspond to the 2.0-kb band seen on blots, or else the parental mRNA(s) contains an extensive 5′ untranslated region not present in these cDNA clones. The relationship of these distinct mRNA species to extracellular DAF forms in body fluids and connective tissue matrix (5) also remains to be determined.

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