

Dr(a-) Polymorphism of Decay Accelerating Factor

Biochemical, Functional, and Molecular Characterization and Production of Allele-specific Transfectants

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Abstract

The Dr^a antigen belongs to the Cromer-related blood group system, a series of antigens on decay accelerating factor (DAF), a glycosyl-phosphatidylinositol-anchored membrane protein that protects host cells from complement-mediated damage. We studied the rare inherited Dr(a-) phenotype to ascertain the associated biochemical and functional changes in DAF and to characterize the basis for this polymorphism. Radioimmunoassay and flow cytometric analysis of Dr(a-) erythrocytes demonstrated 40% of normal surface expression of DAF but normal levels of several other glycosyl-phosphatidylinositol-anchored proteins, distinguishing this phenotype from that of paroxysmal nocturnal hemoglobinuria. Western blots confirmed this reduced DAF expression and indicated a slightly faster mobility of the molecule on SDS-PAGE. Despite the reduced DAF expression, Dr(a-) erythrocytes functioned normally in the complement lysis sensitivity assay. Utilization of the polymerase chain reaction to amplify mononuclear cell genomic DNA from three unrelated Dr(a-) individuals demonstrated that a point mutation underlies the Dr(a-) phenotype: a C to T change in nucleotide 649 resulting in a serine¹⁶⁵ to leucine change. This defines the Dr^b allele of DAF, which can be distinguished from Dr^a by a Taq I restriction fragment length polymorphism. We created transfected Chinese hamster ovary cell lines expressing either the Dr^a or the Dr^b allelic form of DAF. These allele-specific transfectants were tested by inhibition of hemagglutination or flow cytometry and confirmed the specificity of anti-Dr^a alloantisera. The allele-specific transfectants could form the basis of a new serological approach to immunohematology. (*J. Clin. Invest.* 1991. 87:1945-1952.) Key words: complement • erythrocytes • Cromer-related blood group • glycosyl-phosphatidylinositol membrane anchor • flow cytometry

Introduction

Decay accelerating factor (DAF,¹ CD55) is a 70,000 M_r membrane protein that protects host cells from damage by auto-

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1. Abbreviations used in this paper: AST, allele-specific transfectant; CHO, Chinese hamster ovary cell line; CLS, complement lysis sensitivity; DAF, decay accelerating factor; GPI, glycosyl-phosphatidylinositol; LFA, lymphocyte function-associated; PCR, polymerase chain reaction; PNH, paroxysmal nocturnal hemoglobinuria; RFLP, restriction fragment length polymorphism; SCR, short consensus repeat.

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gous complement (reviewed in 1). DAF is one member of the regulators of complement activation gene family, a group of linked genes on the long arm of human chromosome 1 (2, 3) that are comprised of multiple copies of a 60-amino acid short consensus repeat (SCR). The products of these genes all serve to downregulate the activity of the complement system at the level of the C3 convertase. DAF is widely distributed in peripheral blood cells and in epithelial and endothelial tissues (4-7). It acts to prevent the assembly of C3 convertases and to dissociate preformed C3 convertases (8-11).

A novel structural feature of DAF is its attachment to the plasma membrane through a glycosyl-phosphatidylinositol (GPI) anchor (12, 13). The class of GPI-anchored membrane proteins (reviewed in 14, 15) includes the complement-regulatory factors DAF, membrane inhibitor of reactive lysis (16-18), and C8-binding protein/homologous restriction factor (19, 20). The complement-regulatory function of DAF and the GPI anchoring both come into play in the disease paroxysmal nocturnal hemoglobinuria (PNH; reviewed in 21), an acquired hemolytic anemia. The underlying molecular basis of PNH is a failure to express any GPI-anchored proteins on the cell surface, presumably due to a defect in one of the enzymes responsible for synthesis or attachment of the GPI anchor. Therefore the affected blood cells in PNH patients fail to express the GPI-anchored complement-regulatory molecules and are abnormally sensitive to complement, with part of the increased sensitivity due to the absence of DAF in these cells (9, 22, 23).

The cDNA (24, 25) and gene (26) for human DAF have been cloned. Several polymorphisms have been identified in the noncoding regions of the DAF gene by restriction fragment length polymorphisms (RFLP) analysis (3, 27). Recently the Cromer blood group antigens (reviewed in 28) have been shown to reside on the DAF molecule (29), so that the serologically identified Cromer blood group alleles mark polymorphisms in the encoded DAF protein. These Cromer blood group phenotypes thus provide a basis for biochemical and functional investigation of alternate forms of DAF.

One rare Cromer phenotype is Dr(a-), which has been found as a recessive trait in individuals in four unrelated Israeli families that all originally emigrated from the Bukharan region of Russia (30, 31; Levene, C., unpublished data). Erythrocytes of the Dr(a-) phenotype lack expression of the Dr^a antigen and have low expression of all of the other Cromer alleles including Cr^a, Tc^a, Es^a, WES^b, and IFC. No hematological abnormalities have been reported to be associated with the Dr(a-) phenotype.

In this study we investigated the biochemical expression of DAF in the Dr(a-) phenotype and characterized the functional properties of this DAF variant. Furthermore, we have identified the molecular (nucleotide) change that defines the Dr(a-) phenotype, found an allele-specific RFLP that can be used to phenotype individuals, and have created allele-specific transfectants that can be used to identify alloantibodies.

Methods

Antibodies. Two rabbit polyclonal anti-DAF (29, 32) were used for radioimmunoassays, immunofluorescence, immunoprecipitation, and Western blots. Monoclonal antibodies 3.3.136 and K98 (33) (gift of Dr. Robert Knowles, Memorial Sloan Kettering, New York) directed against DAF were also used in radioimmunoassays and immunofluorescence. Monoclonal antibodies against acetylcholinesterase (34) (American Type Culture Collection, Rockville, MD) and CD58 (35) have been previously described. (Anti-CD58 was a gift from Dr. Timothy Springer, Dana Farber Cancer Institute, Boston.) Monoclonal anti-CD59 (2/24D4) was used as part of investigations performed for the Second International Workshop on Monoclonal Antibodies Against Red Cells and Related Antigens (Lund, Sweden, 1990). Polyclonal anti-CD59 has also been previously described (16) and was provided by Dr. Charles Parker, University of Utah, Salt Lake City.

Radioimmunoassay of erythrocyte expression of GPI-anchored proteins. Radioimmunoassays to measure antibody reactivity with erythrocytes from Dr(a+) or Dr(a-) individuals were performed as previously described (36) except that radiolabeled Staphylococcal protein A was used to detect binding of rabbit antisera to DAF and CD59 (29).

Western blots. Western blotting of erythrocyte proteins was performed as previously described (37), except that antibody binding was detected using goat anti-rabbit IgG conjugated with alkaline phosphatase (Promega Biotec, Madison, WI) and a chromogenic substrate (a mixture of 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium) prepared according to manufacturer's instructions.

Complement lysis sensitivity assays. The complement lysis sensitivity (CLS) assay was carried out as described (38). Briefly, erythrocytes were incubated with saturating amounts of human anti-I at 0°C and then incubated with varying dilutions of fresh human serum containing complement at 37°C. Lysis of erythrocytes was measured by spectrophotometric analysis of hemoglobin released into the supernatant fluid. The CLS H₅₀ is defined as the reciprocal of the dilution of serum used divided by the milliliter equivalents of serum required to obtain lysis of 50% of the cells.

Amplification of genomic DNA by PCR. Mononuclear cells were prepared from peripheral blood samples by Ficoll-Hypaque density gradient centrifugation (39). Genomic DNA was prepared from these cells by direct lysis in polymerase chain reaction (PCR) buffer with nonionic detergents and proteinase K as described (40). PCR amplification of the genomic DNA with Taq polymerase was performed in a 50- μ l vol containing 0.2–1.0 μ g DNA, 1 \times PCR buffer (50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin), 200 μ M deoxynucleoside triphosphate mixture, 0.5 μ M of each primer, and 1.5 U Taq DNA polymerase (41). The primers of 17–26 nucleotides were synthesized on a synthesizer (Pharmacia Fine Chemicals, Piscataway, NJ) and were designed based on the sequence of the human DAF gene (26); in general, oligonucleotide pairs were synthesized to match sequences in the 5'- and 3'-introns surrounding either one or several exons. The PCR was done with an initial denaturation of 94°C for 2 min, 35 cycles consisting of denaturation at 94°C for 1 min/annealing at 45–55°C for 1 min/elongation at 72°C for 1–3 min, and a final elongation step at 72°C for 10 min.

DNA sequencing. PCR products were treated with T4 DNA polymerase to repair any possible ragged ends, extracted with phenol-chloroform, precipitated with ethanol, kinased with T4 polynucleotide kinase, and then purified on agarose gels and subcloned into the Eco RV site of the plasmid pBluescript KS+ (Stratagene, La Jolla, CA) by standard techniques (42). The double-stranded plasmid DNA (43) was sequenced with Sequenase DNA polymerase (44). Any differences from wild-type sequence were confirmed on several independent isolates to rule out a PCR or subcloning artifact.

RFLP analysis. PCR products were digested with Taq I restriction endonuclease in the PCR buffer at 65°C for 1.5 h under mineral oil, and then analyzed on an agarose gel with ethidium bromide staining.

Transfections. DAF cDNA was subcloned into the Eco RI site of the expression vector pSFFV.neo (45; a gift of S. Fine and D. Loh,

Washington University) containing the spleen focus-forming virus 5' long terminal repeat, SV40 splicing and polyadenylation signals, and the neomycin-resistance gene. Chinese hamster ovary (CHO) cells were transfected by lipid-mediated DNA transfection using 10 μ g DNA and 100 μ g Lipofectin (46). Positive cells were selected by resistance to the neomycin analogue G418 (0.25 mg/ml active drug) and individual subclones were produced by limiting dilution.

Biosynthetic labeling of cell lines and immunoprecipitations. CHO cell transfectants were plated 24 h before labeling in multiple 100-mm dishes so as to be at 70% confluence at the time of labeling. The cells were washed, incubated in cysteine-free Ham's F-12 medium with 5% dialyzed FCS for 1 h, and then 100 μ Ci/ml [³⁵S]cysteine was added. For kinetic experiments, the cells were pulsed for the indicated time period and then the chase was begun by adding a 10,000-fold excess of unlabeled cysteine. At given time points a plate was processed by washing with cold PBS and solubilizing the cells in 1% Triton X-114/Tris buffered-saline containing the protease inhibitors 2 mM PMSF, 1 μ M pepstatin, and 100 U/ml aprotinin. A detergent extract (47) of the cell lysate was immunoprecipitated with rabbit polyclonal anti-DAF antiserum as described (48) and analyzed on 9% SDS-polyacrylamide gels under reducing conditions followed by fluorography.

Inhibition of hemagglutination. Agglutination of human erythrocytes with human alloantiserum plus antiglobulin (Coombs') reagent (Gamma Biologicals, Houston, TX) was carried out by standard serological techniques. Inhibition of hemagglutination was done by preincubating plasma samples or tissue culture supernates with the alloantiserum for 15 min at 37°C and then proceeding with the hemagglutination reaction as above.

Immunofluorescence. CHO transfectants were removed from tissue culture flasks by incubating for 2 min with trypsin-EDTA; preliminary experiments demonstrated that this brief treatment did not remove surface DAF, consistent with the previously described resistance of DAF to degradation by trypsin (49). Cells were stained with specific antibody followed by FITC-labeled second antibody and analyzed by flow cytometry to assess surface expression of DAF (50). Because human alloantisera gave high backgrounds on this analysis, the human alloantibodies were first partially purified from serum by absorption and elution from human erythrocytes using Elu-Kit II according to the manufacturer's directions (Gamma Biologicals).

Results

DAF expression on Dr(a-) erythrocytes. Dr(a-) erythrocytes showed reduced hemagglutination with antibodies to non-Dr^a Cromer-related blood group antigens (30, 31), suggesting a reduced level of all Cromer-related antigens and hence of DAF. To confirm this directly, a radioimmunoassay was used to measure the levels of anti-DAF reactivity with Dr(a+) and Dr(a-) erythrocytes. As shown in Table I, erythrocytes from a Dr(a-) individual demonstrated reduced reactivity with both a rabbit polyclonal and one monoclonal anti-DAF (3.3.136), averaging ~40% of normal in each case. Interestingly, a second monoclonal anti-DAF (K98) showed almost no reactivity with Dr(a-) erythrocytes but reacted strongly with Dr(a+) erythrocytes, suggesting that this monoclonal antibody might recognize an epitope at or near the polymorphic amino acids corresponding to the Dr(a-) phenotype.

Measurements of surface DAF expression were also made by immunofluorescence. Erythrocytes from two additional Dr(a-) and two Dr(a+) individuals were treated with a different rabbit polyclonal anti-DAF and FITC-labeled second antibody and assessed by flow cytometry. The two Dr(a-) erythrocytes yielded mean channel fluorescence values that were 32% and 48% of the average values for the Dr(a+) erythrocytes (data not shown).

Table I. Reactivity of Normal and Dr(a-) Erythrocytes with Antibodies to DAF

Cell type	Specific cpm bound		
	Polyclonal Rabbit anti-DAF	Monoclonal	
		3.3.136	K98
Normal 1	2945	5246	3181
Normal 2	3149	5566	4797
Normal 3	3406	4980	4009
Dr(a-)	1488	2034	258

Erythrocytes from normal, i.e., Dr(a+), donors or a Dr(a-) individual were treated with anti-DAF antibodies followed by appropriate radiolabeled second antibody or Staphylococcal protein A. Specific counts bound was calculated as the difference in bound radiolabel between test antibody and a negative control antibody (normal rabbit serum or a nonspecific monoclonal antibody).

To further characterize the DAF membrane protein from Dr(a-) erythrocytes, Western blots were done with rabbit polyclonal anti-DAF. Western blots consistently demonstrated the markedly reduced expression of DAF by Dr(a-) erythrocytes (Fig. 1). The apparent M_r of DAF on Dr(a-) erythrocytes, as judged by SDS-PAGE, was approximately the same as on Dr(a+) erythrocytes; however, comparison of the two species on gels of differing polyacrylamide concentrations suggested a possible small decrease (< 2,000) in apparent M_r of the Dr(a-) DAF (not shown).

Expression of GPI-anchored proteins on Dr(a-) erythrocytes. Having seen that Dr(a-) erythrocytes express a reduced amount of a DAF species possessing near normal size, we next studied expression of other GPI-anchored membrane proteins to ascertain whether the defect was limited to DAF. Radioimmunoassay was used to measure reactivity with antibodies to three other GPI-anchored proteins: acetylcholinesterase, membrane inhibitor of reactive lysis (MIRL or CD59), and lymphocyte function-associated antigen 3 (LFA-3 or CD58). The Dr(a-) erythrocytes showed a markedly reduced reactivity with antibodies to DAF, but they showed essentially normal reactivity with antibodies against the other three GPI-anchored proteins (Table II). By contrast, when cells from a patient with

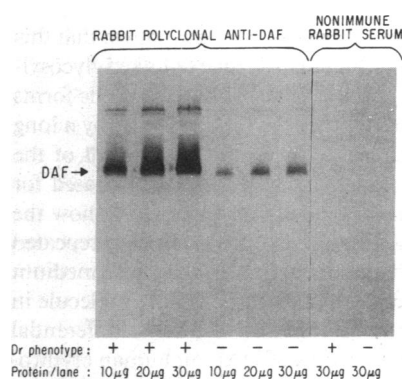


Figure 1. Western blot for DAF in human erythrocytes. Varying amounts of erythrocyte membranes (protein/lane) prepared from Dr(a+) or Dr(a-) individuals were subjected to Western blot analysis with rabbit polyclonal anti-human DAF or control nonimmune rabbit serum. The arrow marks the band corresponding to DAF. A

minor band of approximately twice the M_r of DAF is also visualized; this DAF-2 species has been reported previously (56, 57).

Table II. Comparison of Reactivity of Dr(a-) and PNH III Erythrocytes with Antibodies to GPI-linked Membrane Proteins

Antibody type and specificity	Percentage of normal binding	
	Dr(a-)	PNH III
Polyclonal anti-DAF	45%*	15%*
Monoclonal 3.3-136 anti-DAF	39%*	24%*
Monoclonal K98 anti-DAF	6%*	10%*
Polyclonal anti-AChE	98%	ND
Monoclonal anti-AChE	95%	5%*
Polyclonal anti-CD59	99%	23%*
Monoclonal anti-CD59	100%	ND
Monoclonal anti-CD58 (LFA-3)	88%	39%*

The reactivity of various antibodies against GPI-linked membrane proteins with erythrocytes from normal donors, a Dr(a-) individual, or a PNH III individual was measured as specific counts bound as described in the legend to Table I. The results are listed as a percentage of the counts for normals.

* Indicates that difference is statistically significant, compared to normal Dr(a+) erythrocytes.

PNH were tested, they showed significantly reduced expression of all four GPI-anchored proteins, consistent with the known failure of surface expression of all GPI-anchored membrane proteins in PNH erythrocytes. These data clearly demonstrated the different phenotypes of Dr(a-) and PNH erythrocytes.

CLS assay of Dr(a-) erythrocytes. Next the Dr(a-) erythrocytes were tested for functional activity of DAF. DAF serves to protect cells from lysis by complement by inhibiting formation and accelerating the decay of the C3 and C5 convertases (8-11), thus preventing the complement cascade from proceeding to final hemolysis. This was tested by the CLS assay of Rosse and Dacie (38). Dr(a-) erythrocytes gave a normal result in this assay, with values for CLS H_{50} ranging from 1.35 (Fig. 2) to 1.8, values well within the normal range. Even with reduced expression of DAF, the Dr(a-) erythrocytes had no functional defect measured by this assay.

Single nucleotide change underlies Dr(a-) phenotype. The next step in the molecular characterization of the Dr(a-) phenotype was to identify the amino acid change or changes that

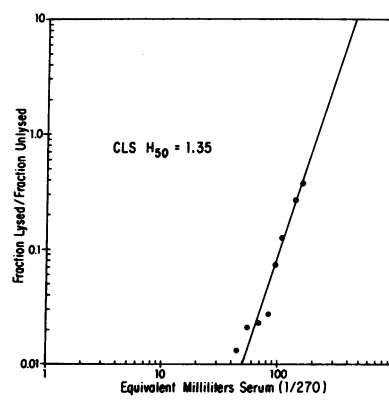


Figure 2. CLS assay of Dr(a-) erythrocytes. Erythrocytes from a Dr(a-) individual were incubated with antibody and varying dilutions of fresh human serum complement. The percent hemolyzed cells was measured by spectrophotometer, and the ratio of lysed to unlysed cells was then calculated and plotted on the y axis versus equivalent

milliliters serum on the x axis. The value of CLS H_{50} was found as described in Methods; in several experiments this value ranged from 1.35 to 1.8, all within the normal range.

determine the phenotype, including the antigenicity of the protein. This was accomplished by using PCR amplification to clone and sequence the coding region of membrane DAF. We had previously cloned the human DAF gene, which spans ~ 40 kb and consists of 11 exons (26). The membrane form of DAF is encoded on nine exons (the two additional exons are the first exon encoding the 5'-untranslated region and signal peptide and an alternatively spliced exon that is absent from the mRNA encoding membrane DAF; 25, 26). All of the exons encoding membrane DAF were amplified by PCR from genomic DNA of the original Dr(a-) propositus M.D. (30), and the PCR DNA products were subcloned into the vector pBlue-script KS+ and sequenced. There was only a single nucleotide change in the entire coding region, and this is shown in Fig. 3: nucleotide 649 (numbering per reference 1) changes from C to T in Dr(a-) corresponding to a serine to leucine change at amino acid residue 165 which is in the third SCR.

Since the entire DNA sequence encoding the membrane DAF protein had been sequenced, the single nucleotide change found should correspond to the Dr(a-) phenotype. To confirm this finding, DNA from the identified region was amplified and sequenced in two unrelated Dr(a-) individuals, and both showed the same nucleotide change as the propositus. This nucleotide change thus identifies these Dr(a-) phenotype individuals as homozygous for a specific, identifiable allele of DAF which we designate Dr^b, a low-frequency antithetical allele to the high-frequency DAF allele Dr^a.

Taq I RFLP. The Dr^b change alters the DAF nucleotide sequence 648-651 from TCGA to TTGA, thus removing a Taq I restriction site (see bottom of Fig. 3). This allele-specific RFLP can be used to type individuals at the Dr locus, and this is demonstrated in Fig. 4. A PCR-amplified DNA fragment of ~ 1,250 bp was produced from genomic DNA of either Dr^a or Dr^b individuals. This DNA was digested with Taq I and ana-

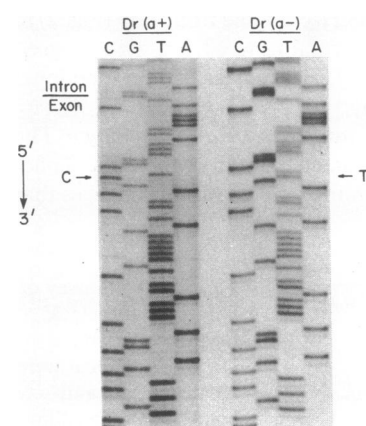


Figure 3. Single nucleotide difference between Dr(a+) and Dr(a-) in exon encoding SCR 3B. PCR was used to amplify genomic DNA of Dr(a+) and Dr(a-) individuals. The 5'-primer was a 20-mer from exon 4 (5'-AGATTGATG-TACCAGGTGGC-3') and the 3'-primer was a 24-mer from intron 5 (5'-GAGTACT-CAGCCTCACAATCT-GAG-3'), thus amplifying the entire exon 5 which encodes SCR 3B. This autoradiograph of the sequencing gel shows the single nucleotide change from C to T in this exon. The se-

quence of the two alleles of DAF from the region surrounding the polymorphic nucleotide is shown below, with that base marked with an asterisk. The Taq I restriction endonuclease site that is present in the Dr(a+) sequence but absent in the Dr(a-) allele is marked with an overline.

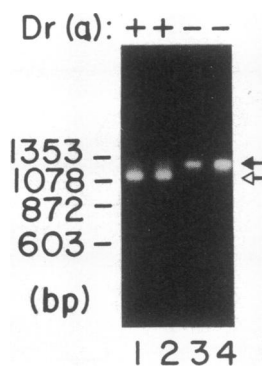
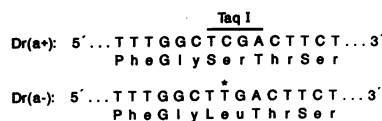


Figure 4. RFLP phenotyping of Dr(a+) versus Dr(a-) individuals. PCR-amplified genomic DNA including the Dr(a+)/Dr(a-) polymorphic locus in exon 5 was produced as described in Fig. 3. Products of the PCR reaction from two individuals each of the Dr(a+) and Dr(a-) phenotypes migrated identically on agarose gels (not shown). The PCR DNA was digested with Taq I and analyzed by agarose gel electrophoresis, and a photograph of the ethidium bromide stained gel is shown. The solid arrow marks the band from Dr(a-) individuals (lanes 3 and 4) with no Taq I sites, whereas the open arrow marks the band from Dr(a+) individuals (lanes 1 and 2) that is produced by Taq I digestion (which also produces a smaller band of 116 bp that is not visualized on this gel). Molecular size markers are shown on the left.

lyzed by agarose gel electrophoresis and ethidium bromide staining, demonstrating a single Taq I site in Dr^a DNA producing fragments of ~ 1,150 and 100 (the latter not visualized on this gel) and the Dr^b DNA with no Taq I site.

Allele-specific transfectants. For further analysis of this DAF polymorphism, we created allele-specific transfectants (AST). Just as allele-specific oligonucleotides permit the analysis of antigen or allele specificity (51), AST will permit the analysis of antibody specificity (see data below and Discussion). We had previously cloned the full-length (wild-type) DAF cDNA (24) that corresponds to the Dr^a allele. The cDNA for the Dr^b allele was created by using oligonucleotide-directed in vitro mutagenesis (52) to introduce the single change at nucleotide 649 (C to T) into the Dr^a cDNA. Both the Dr^a and the Dr^b allelic cDNA were subcloned into the expression vector pSFFV.neo (45) and transfected into CHO cells. Permanent transfectants were selected in the neomycin analogue G418. Individual subclones were produced by limiting dilution followed by screening for surface DAF expression by flow cytometry, and one high-expressing cell line of each allelic type was selected.

Biosynthesis and decay kinetics of DAF in AST. The biosynthesis of DAF in the Dr^a and Dr^b AST was studied in standard pulse-chase kinetic experiments. A short [³⁵S]cysteine pulse followed by a chase period demonstrates essentially identical kinetics for the pro-DAF species chasing into the higher M_r mature DAF species (Fig. 5 A). Previous work has shown that this large M_r shift is mainly the result of extensive O-linked glycosylation (48). Next, to look at the stability of the two allelic forms of DAF, the AST were biosynthetically radiolabeled by a long pulse, chased in nonradioactive medium, to allow all of the intracellular DAF to get to the surface, and then chased for various time periods. The two allelic forms of DAF show the same decay kinetics (Fig. 5 B). This experiment was repeated with the addition of 20% human serum to the chase medium (for the possible addition of any protease or other molecule in human serum that might be responsible for the differential stability of the polymorphic forms of DAF on human erythrocytes circulating in blood), but again there was no significant difference between the two DAF species (not shown). Thus, there is no evidence from these kinetic experiments in CHO

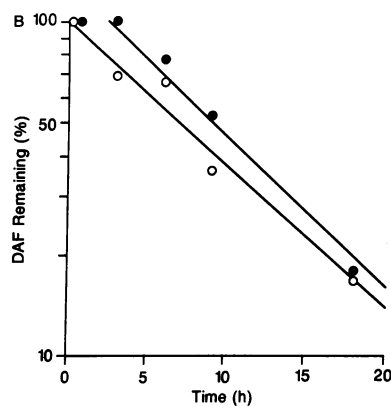
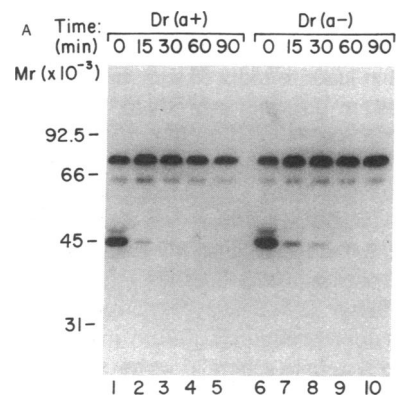


Figure 5. Kinetic analysis of DAF biosynthesis and decay in AST. (A) The Dr(a+) and Dr(a-) (Dr^a and Dr^b, respectively) AST were biosynthetically labeled with [³⁵S]cysteine for 15 min and then chased for the indicated time periods. Cell lysates were prepared and immunoprecipitated with anti-DAF and analyzed by SDS-PAGE. The fluorograph demonstrates the same kinetics in each allele for processing of the intracellular pro-DAF species of 45 kD to the mature DAF species of 74 kD. (B) The kinetics of DAF decay were assessed by a 2-h biosynthetic label followed by a 2-h chase to permit all DAF to be processed to the mature DAF species and transported to the cell surface. Cell lysates were prepared at this $t = 0$

point and subsequent time points and analyzed as described above. Laser scanning densitometry of the fluorograph was used for the quantitation that is depicted in the graph (○, Dr^a; ●, Dr^b). Linear regression analysis yielded a $t_{1/2}$ value of 7.0 h for Dr^a and 6.6 h for Dr^b.

AST to explain the difference in the level of expression of the two Dr allelic forms of DAF in human erythrocytes.

Inhibition of anti-Dr^a hemagglutination by AST. Since there was only a single nucleotide change in the coding region of the Dr^b allele of DAF, it was clearly implicated in the Dr polymorphism. In order to confirm this finding, we tested the DAF AST for reactivity with anti-Dr^a alloantibodies, the reagents used to define the actual polymorphism. Since the alloantibodies are normally identified in standard blood bank hemagglutination reactions, we used an inhibition of hemagglutination test. Material from the AST were used to inhibit alloantibody to Dr^a, which was then assayed by hemagglutination. As seen in Table III, the Dr^a AST inhibited anti-Dr^a but neither the Dr^b AST nor the control cell transfected with vector alone caused any inhibition. As further controls, material from the HeLa cell line or plasma from a Dr^a individual caused inhibition but plasma from a Dr^b individual did not cause inhibition of hemagglutination, as expected.

Flow cytometric testing of AST. We also tested the AST in a direct flow cytometric analysis. Specifically, the alloantibodies were used to sensitize the AST, which were then stained with FITC-labeled second antibody and analyzed by flow cytometry. The results in Fig. 6 demonstrate that the AST show the expected Dr specificity, i.e., the alloanti-Dr^a recognizes the Dr^a but not the Dr^b AST, whereas the rabbit polyclonal anti-DAF recognizes both AST. Thus, the AST testing allows us to con-

firm the single amino acid change from Dr^a to Dr^b as the basis of the Dr polymorphism.

Discussion

This study has analyzed at the biochemical, functional, and molecular level the properties of a DAF polymorphic variant. This has produced several conclusions concerning DAF, and it has also led to a new approach to serological analysis in immunohematology through the use of allele-specific transfectants. At the protein level, the Dr(a-) phenotype was found to be due to reduced surface expression of a slightly altered form of DAF (based on its mobility on SDS-PAGE), with normal expression of other GPI-anchored membrane proteins. This form of DAF possesses normal complement regulatory activity as measured in the CLS assay. The actual molecular basis for the phenotype is a variant allele of DAF, named Dr^b, that encodes a single nucleotide (and amino acid) change from the common Dr^a allele. Finally, these two alleles of DAF were differentiated by an allele-specific Taq I RFLP, and were expressed in foreign cells to create AST that could be used in analyzing the specificity of human alloantiserum.

The Dr(a-) phenotype of DAF is one of eight identified genetic variants in the Cromer-related blood group system that resides on the DAF molecule (29). With the exception of the Inab phenotype, which lacks all surface expression of DAF, the Dr(a-) phenotype is the only variant that demonstrates reduced DAF expression by hemagglutination. Because the PNH phenotype results in reduced expression of all GPI-anchored membrane proteins, it was important to compare these two phenotypes. Radioimmunoassays measuring antibody reactivity against erythrocytes clearly demonstrated that the Dr(a-) erythrocytes had an isolated defect in expression of DAF with normal expression of three other GPI-anchored proteins. This is in marked contrast to the PNH III phenotype with reduced expression of all of these GPI-anchored proteins (Table II). Western blot analysis confirmed the reduced expression of DAF protein, and suggested a slight alteration of SDS-PAGE mobility.

Previous investigations of the Inab phenotype had found a mild defect in complement regulatory activity as judged by the complement lysis sensitivity assay, comparable to PNH II cells (53, 54). It was therefore of interest to test Dr(a-) erythrocytes,

Table III. Inhibition of Anti-Dr^a Hemagglutination by AST

Sample	Inhibition
CHO/Dr ^a	+
CHO/Dr ^b	-
CHO/SFFV.neo	-
HeLa	+
Dr ^a plasma	+
Dr ^b plasma	-

Tissue culture supernatants from CHO cells transfected with Dr^a or Dr^b allele DAF cDNA or the expression vector SFFV.neo or from the HeLa cell line, or plasma of Dr^a or Dr^b homozygous individuals were incubated with alloantibody to Dr^a, then tested for hemagglutination of normal erythrocytes. Inhibition (+) or failure to inhibit (-) hemagglutination is scored.

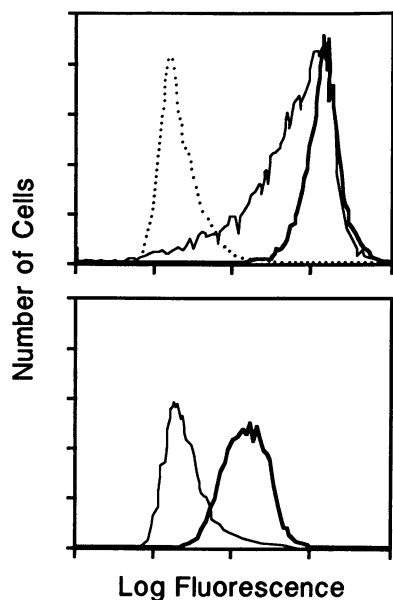


Figure 6. Assay using AST to assess alloantisera specificity by flow cytometry. The Dr^a and Dr^b AST were treated with human anti- Dr^a or with rabbit anti-human DAF, followed by appropriate FITC-labeled second antibody. They were analyzed by flow cytometry, and the panels show relative number of cells versus relative log fluorescence on a four decade scale. (Top) Dr^a AST (thick line) and Dr^b AST (thin line) both show strong DAF expression as assessed with rabbit anti-human DAF compared to the negative control CHO

cells transfected with vector alone (dotted line). (Bottom) Testing with human anti- Dr^a demonstrates reactivity with the Dr^a AST (thick line) but not the Dr^b AST (thin line).

which might have altered complement regulatory function either because of the reduced expression ($\sim 40\%$ of normal) or because of the underlying minor structural alteration in the DAF molecule. In fact, the $Dr(a-)$ cells had a normal CLS titer (Fig. 2). Thus, neither the structural change nor the greater than twofold reduction in expression impair DAF function (at least as judged by this assay). This suggests that there might normally be an excess of required DAF on the surface of erythrocytes.

The remaining part of these studies focused on the actual molecular variation that underlies the Dr polymorphism: a single nucleotide change from C to T resulting in a single amino acid change from serine to leucine. This was the only nucleotide change present in the coding region of the DAF gene of the original $Dr(a-)$ proband, and the same change was found in two other unrelated $Dr(a-)$ individuals. The presence of this nucleotide sequence can then be used to identify the DAF allele present in these individuals, which we designate Dr^b . This is the first case, to our knowledge, where an erythrocyte antigen was first defined at the nucleotide level and not by the discovery of an alloantiserum of the appropriate specificity. This method of antigen identification at the DNA level will be especially useful for low-frequency alleles where it is very unlikely that an alloantiserum will be found.

Expression of the two Dr alleles in CHO cells to create AST has allowed us to probe the properties conferred by the allelic difference, as well as to set up a system for alloantibody identification (see below). The pulse-chase experiments showed no difference between the two allelic forms of DAF as far as the kinetics of processing of DAF from a precursor form to the mature, glycosylated form or in the kinetics of decay. Therefore, the polymorphic difference as tested in these kinetic experiments in CHO transfectants does not explain the reduced expression of DAF in Dr^b erythrocytes. There are two broad classes of explanations for this finding: either the reduced ex-

pression does not map to the same variation causing the allelic difference (e.g., there is a second change, perhaps in the regulatory region of the gene, that leads to reduced transcription), or the CHO transfectant system does not reproduce the conditions causing the reduced expression in human erythrocytes. Since two separate, widely spaced mutations seem less likely a priori, one has to consider the many differences that arise in comparing the CHO cells and the erythrocytes. Some of the major considerations are: (a) the altered amino acid might result in a change in posttranslational processing (since it is a serine to leucine change, loss of *O*-linked glycosylation is possible) in human cells that is not reproduced faithfully in the CHO cells; (b) the tissue culture system in general does not match the in vivo blood stream conditions; and (c) the CHO cell type (and perhaps all nucleated cells) is not a good model for the erythrocyte as far as DAF expression and stability. In regard to this latter point, it should be noted that the reduced expression of Dr^b DAF has only been documented on human erythrocytes, so investigation of human leukocytes should also be an avenue for future study of this question.

The final impact of the studies reported here is in the area of serological analysis that forms part of the foundations of immunohematology. Through the use of allele-specific RFLP analysis we can phenotype individuals at the DAF Dr polymorphic locus (Fig. 4). This was made possible in the present situation because the polymorphism involved the loss of a *Taq* I restriction endonuclease site, but if this were not available the DNA sequence difference could be ascertained by hybridization with allele-specific oligonucleotides. That approach has been used extensively for typing polymorphic HLA loci (55).

The more common problem in immunohematology than the identification of alloantigen specificity (phenotyping) is the identification of alloantibody. The system that we have established with AST allows that alloantibody analysis as demonstrated by either of two assays, inhibition of hemagglutination (Table III) or flow cytometry (Fig. 6). In the present case they allowed us to come full circle and confirm the molecular basis of the polymorphism using the alloantisera to Dr^a that originally defined the polymorphism. In the general case they would allow serological analysis of any alloantiserum. One of the major advantages of this approach using AST is that only a single allele of one blood group system is expressed by each AST in a foreign cell background that lacks all of the other blood group antigens of that or other systems. Therefore one can test an alloantiserum that might contain multiple erythrocyte alloantibodies (and possibly also an autoantibody) against an AST and get an answer regarding the specific allele of that AST. Similar testing against human erythrocytes is ambiguous because the erythrocytes express multiple alloantigens. As the major human blood group systems are cloned, one could create a panel of AST covering most or all of the clinically important systems, allowing identification of all of the specificities in a complex alloantiserum. Neither of the two assays that were used in this study would be the most practical for routine use in serological analysis in the clinical laboratory, but they could be replaced with other methods of testing the alloantiserum versus the AST, such as agglutination reactions or some form of enzyme-linked immunoassay. The actual demonstration in this report that one can use allele-specific RFLP and allele-specific transfectants for the analysis of erythrocyte alloantigens and alloantibodies, respectively, opens up a new molecular approach to immunohematology.

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