

Molecular characterization of sialophorin (CD43), the lymphocyte surface sialoglycoprotein defective in Wiskott–Aldrich syndrome

(immune deficiency/T-cell activation/gene mapping/transient expression)

C. SIMON SHELLEY*†, EILEEN REMOLD-O'DONNELL*†, ALVIN E. DAVIS III*, GAIL A. P. BRUNS‡, FRED S. ROSEN*†, MICHAEL C. CARROLL*, AND ALEXANDER S. WHITEHEAD*

Divisions of *Immunology and †Genetics, Children's Hospital, the Departments of Pediatrics, Biological Chemistry and Molecular Pharmacology, & Pathology, Harvard Medical School, and ‡The Center for Blood Research, 800 Huntington Avenue, Boston, MA 02115

Communicated by Emil R. Unanue, January 9, 1989

ABSTRACT Sialophorin (CD43) of leukocytes and platelets is a surface sialoglycoprotein that is phenotypically defective on lymphocytes of patients with the X chromosome-linked immunodeficiency Wiskott–Aldrich syndrome. Previous studies with monoclonal antibodies indicate that sialophorin is a component of a T-lymphocyte activation pathway. Here we describe the cDNA cloning and derived amino acid sequence of human sialophorin. The sequence predicts an integral membrane polypeptide with an N-terminal hydrophobic signal region followed by a mucin-like 235-residue extracellular region with a uniform distribution of 46 serine, 47 threonine, and 24 proline residues. This is followed by a 23-residue transmembrane region and a 123-residue C-terminal intracellular region. These latter regions have been highly conserved during evolution; the intracellular region contains a number of potential phosphorylation sites that might mediate transduction of activation signals. The chromosomal location of the sialophorin gene was determined and the implications of this assignment for the pathogenesis of the Wiskott–Aldrich syndrome are discussed.

Wiskott–Aldrich syndrome is an X chromosome-linked immunodeficiency characterized by thrombocytopenia with platelets of reduced size and function, progressive deterioration of T-lymphocyte function, eczema, and inability to produce antibodies to carbohydrate antigens (1, 2). Partial engraftment of donor T cells during allogeneic bone marrow transplantation completely corrects the immunodeficiency state, demonstrating that the immunodeficiency is a primary T-lymphocyte defect (3). In an earlier study, we reported that Wiskott–Aldrich syndrome is associated with a defect of sialophorin, a leukocyte/platelet surface molecule (4). Sialophorin, which has also been called gpL115 (4), LSGP (5), leukosialin (6), and CD43 (7), is a heavily glycosylated mucin-type (acidic) glycoprotein (>50% carbohydrate) with one N-linked and multiple O-linked carbohydrate units (8). Cell-type specific glycosylation patterns give rise to two forms of sialophorin with apparent molecular weights of 115,000 and 135,000 (9). Sialophorin is expressed early in thymus ontogeny and, therefore, may play a role in the regulation of T-cell maturation and the development of the thymic repertoire. It has been suggested that the maintenance of lymphocytes in circulation (10), as well as their surface morphology (11), is dependent on the presence on the cell surface of intact native sialophorin molecules bearing a high density of negative charge. In addition, monoclonal antibodies to sialophorin potentially activate human T lymphocytes independently of the T-cell receptor–CD3 complex, suggesting that sialophorin is a critical component of an independent T-lymphocyte activation

pathway (ref. 12 and L. B. Silverman and T. Chatila, personal communication). Studies with phorbol 12-myristate 13-acetate indicate that sialophorin is subject to phosphorylation by protein kinase C (13). Assuming that sialophorin is involved in a T-cell receptor/CD3-independent T-cell activation pathway, phenotypic abnormalities of this molecule may underlie the immunodeficiency observed in the Wiskott–Aldrich syndrome. To further investigate sialophorin function in normal lymphocytes and its dysfunction in Wiskott–Aldrich syndrome, we have cloned and sequenced[§] a cDNA for human sialophorin and have determined the chromosomal location of the gene.

MATERIALS AND METHODS

Protein Purification and Generation of Tryptic Peptides and Protein Sequence. Asialo-sialophorin was purified from CEM lymphoblastoid cells (8). Peptides were prepared by incubating 100–300 μ g of asialo-sialophorin with 2–4% (wt/wt) L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at 37°C for 18 hr followed by C₁₈ reverse-phase HPLC. N-terminal sequence of the peptides was determined by automated Edman degradation using a Beckman model 890C sequencer modified by a cold tap (14) or an Applied Biosystems model 477A protein sequencer.

Identification and Characterization of Sialophorin-Specific cDNA Clones. A 45-base oligonucleotide probe, corresponding to 15 amino acids of a sialophorin tryptic peptide, was synthesized using an Applied Biosystems model 380B DNA synthesizer. The probe was radiolabeled with ³²P by polynucleotide kinase (New England Biolabs) and used to screen a CEM cell cDNA library constructed in λ gt11 (gift of R. Finberg, Dana–Farber Cancer Institute, Boston) (15). Hybridizing clones were isolated and their inserts were excised with *Eco*RI and subcloned into pBR322. The inserts were used to screen a HPB-ALL T-lymphoblastoid cell cDNA library constructed in the high-efficiency expression vector π H3M (16) to obtain full-length cDNA clones. Similar screening of an EMBL3 library (gift of S. Orkin, Children's Hospital, Boston) yielded a genomic clone containing a 20-kilobase (kb) insert; a 1.8-kb *Pvu* II subfragment was subcloned into the *Hinc*II site of pAA-PZ618 (Gold Biotechnology, Saint Louis) to generate the clone GEN1.8. DNA sequencing was performed by either chemical cleavage (17) or dideoxynucleotide chain termination (18).

RNA and DNA Blot Analysis. Total RNA was isolated from actively proliferating cells (19); poly(A)⁺ RNA was selected by oligo(dT) chromatography (20) and size-fractionated by formaldehyde/agarose electrophoresis (21). Total genomic DNA was isolated from human, mouse, hamster, and somatic hybrid cells by lysis in 1% Triton X-100 then overnight

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[§]The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04536).

incubation at 37°C with 0.5% NaDodSO₄ and proteinase K (0.5 mg/ml) followed by phenol/chloroform extraction (22). After cleavage with restriction endonucleases DNA was subjected to agarose gel electrophoresis. RNA and DNA blots on nitrocellulose (Schleicher & Schuell) were prepared and hybridized with radiolabeled sialophorin-specific cDNA probes (15, 23).

COS Cell Transfection. COS cells at 50% confluence in 15-cm dishes were transfected using DEAE-dextran (31). Transfected and untransfected COS cells, CEM cells, and HPB-ALL cells were pulse-labeled for 10 min with [³⁵S]methionine, lysed, and immunoprecipitated with rabbit anti-sialophorin antiserum (9). Immunoblot analysis was performed by subjecting lysates of 10⁶ cells (0.5 × 10⁶ CEM cells) to NaDodSO₄/PAGE, electrophoretic transfer to nitrocellulose, and treatment with monoclonal antibody L10 (10) and with immunoperoxidase detection reagents (24).

Analysis of Amino Acid Sequences. A computer search of homology was performed by using version 16 of the National Biomedical Research Foundation data base (1988). Percentage identities between the human and rat sialophorin protein sequences were calculated using the ALIGN program (25).

RESULTS AND DISCUSSION

Isolation and Characterization of Sialophorin cDNA Clones. Human asialo-sialophorin was isolated from the T-lymphoblastoid cell line CEM and amino acid sequences for five tryptic peptides totaling 50 residues were determined. A 45-base oligonucleotide corresponding to the most frequently used codons (26) specifying the 15 amino acids of one peptide was used to screen a CEM cDNA library. Two clones, CEM0.8 and CEM1.7, with inserts of 0.8 and 1.7 kb, respectively, were obtained. These clones did not represent the entire sialophorin mRNA (presented below); therefore, CEM1.7 was used to screen a HPB-ALL (T lymphoblastoid) cDNA library (16) and two additional clones, HPB1.9 and HPB2.5, were isolated.

The HPB1.9 insert contains 92 nucleotides of a 5'-untranslated region, an open reading frame specifying 400 amino acids (including all five sequenced sialophorin tryptic peptides), a 3' noncoding region of 587 nucleotides, and a "AATAAA" polyadenylation signal. The inserts of

CEM0.8 and CEM1.7 are identical to the 3' 0.8 kb and 1.7 kb, respectively, of HPB1.9 except for a single neutral nucleotide substitution in the coding region and the presence of poly(A) tails. The HPB2.5 insert is identical to the entire HPB1.9 insert except for six positions within a 126-nucleotide stretch of the 3' noncoding region. This difference may be due to differential splicing of a single gene or to allelic variation. HPB2.5 also differs in having 0.6-kb additional 3' sequence extending beyond the 3' noncoding region defined by clones HPB1.9, CEM0.8, and CEM1.7.

Sialophorin mRNA. Northern blot analysis using a fragment of the CEM1.7 insert as a probe revealed species of 1.9 kb and 4.3 kb in poly(A)⁺ RNA from both CEM and HPB-ALL cells (Fig. 1A, lanes 1 and 2). Similar analysis using the 3' noncoding sequence unique to clone HPB2.5 detected only the 4.3-kb species in both cell lines (Fig. 1A, lanes 3 and 4). Partial sequencing (data not shown) of a 1.8-kb fragment of a sialophorin genomic clone (GEN1.8; Fig. 1B) demonstrated that the 3' noncoding sequence unique to HPB2.5 is a direct continuation of the 3' noncoding region of HPB1.9. Therefore, the difference in length between the 1.9-kb and 4.3-kb sialophorin transcripts is due, at least in part, to alternative polyadenylation site utilization. Differential polyadenylation has been described for mRNAs encoding other functional T-cell surface molecules, including CD2 (27) and β₂-microglobulin (28).

The cDNA sequence and mRNA hybridization results indicate that CEM and HPB-ALL cells contain two mRNA species with a common sialophorin-coding region. CEM and HPB-ALL cells express sialophorin of M_r 115,000 and M_r 135,000, respectively (24); therefore, the cDNA sequencing supports the proposal based on co-electrophoresis (6, 9) and peptide mapping (9) that, although different cells express sialophorin of different molecular weight, there is only a single sialophorin polypeptide.

Analysis of Derived Sialophorin Amino Acid Sequence. The sialophorin clones encode a polypeptide of 400 amino acids (Fig. 2). The ATG triplet specifying the N-terminal methionine is flanked by guanosine at nucleotides +1 and -3, in agreement with consensus sequences near the translation start site of eukaryotic mRNAs (29). Immediately after the N-terminal methionine is a hydrophobic region that is presumed to be the signal peptide. Based on known signal

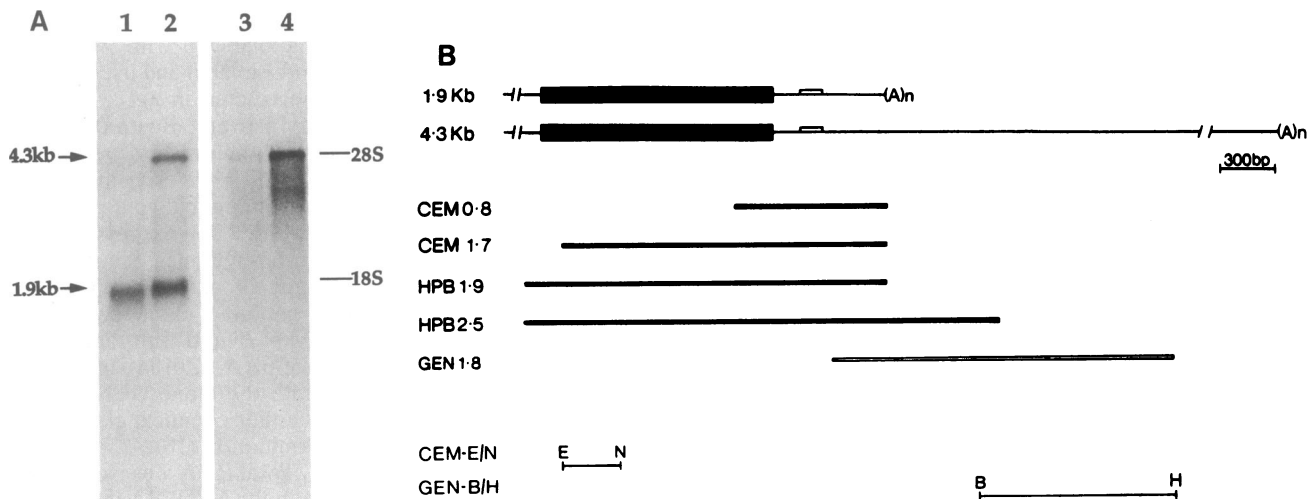


FIG. 1. (A) Northern blot analysis of poly(A)⁺ RNA (5 μg) from CEM cells (lanes 1 and 3) and HPB-ALL cells (lanes 2 and 4). Blots were probed with either CEM-E/N, an *EcoRI*-*Nco*I restriction fragment prepared from the CEM1.7 insert (lanes 1 and 2) or GEN-B/H, a *Bst*XI-*Hind*III restriction fragment from the insert of GEN1.8 (lanes 3 and 4) (15). The positions of 28S and 18S rRNA and the sizes of the sialophorin transcripts are indicated. (B) Organization of sialophorin mRNAs and cDNA. Sialophorin mRNA transcripts of 1.9 and 4.3 kb are shown. Solid boxes represent protein coding regions; lines indicate noncoding regions; and open boxes indicate the region with the six nucleotides that differ in the cDNA inserts. Clones corresponding to the extreme 5' end of the mRNA have not been isolated. The lower part of the figure represents the cDNA clones (solid bars), the genomic clone (open bar), and the probes CEM-E/N and GEN-B/H.

peptidase specificity, cleavage could occur after Gly-19 or Ala-23 (30). There is, however, no firm evidence for cleavage of this peptide. Definitive identification of the N-terminus of the mature sialophorin was not obtained, as repeated attempts to determine N-terminal amino acid sequence for the intact isolated molecule were unsuccessful.

Assuming cleavage after residue 19, the mature polypeptide consists of 381 amino acids with a molecular weight of 38,444 and amino acid composition of Asn₇Asp₁₂Gln₈Glu₂₃His₃Lys₁₁Arg₁₆Ala₃₀Gly₄₂Tyr₂Val₂₈Ile₇Leu₂₈Phe₄Met₉Trp₃Pro₃₈Ser₅₇Thr₅₃. The molecule contains no cysteine. The derived sialophorin protein sequence has three distinct regions: an N-terminal 235-amino acid extracellular region, a 23-residue hydrophobic transmembrane region, and a 123-residue intracellular region. The location of the site(s) that is phosphorylated by protein kinase C (13) cannot be identified among the 17 intracellular serine and threonine

residues; however, Thr-285, Ser-291, Ser-337, Ser-351, and Ser-363, being adjacent to basic residues (32, 33), are the more likely candidates.

Glycosylation of the Extracellular Region. The extracellular region is extremely rich in serine and threonine (93 residues) and proline (27 residues), distributed more or less uniformly throughout the 235-residue region. Compositional data (8), recalculated using a polypeptide of *M*_r 38,444, indicate that mature sialophorin contains one N-linked carbohydrate unit and approximately 84 O-linked units, the latter predominantly sialylated Gal-GalNAc. Thus, approximately 84 of the 93 extracellular serines and threonines carry O-linked units and the remainder are unglycosylated. The N-linked carbohydrate must be located at Asn-239, the single deduced N-glycosylation site. The O-linkage sites cannot be precisely localized since the only known requirement for O-glycosylation is that the sites be extracellular serines or thre-

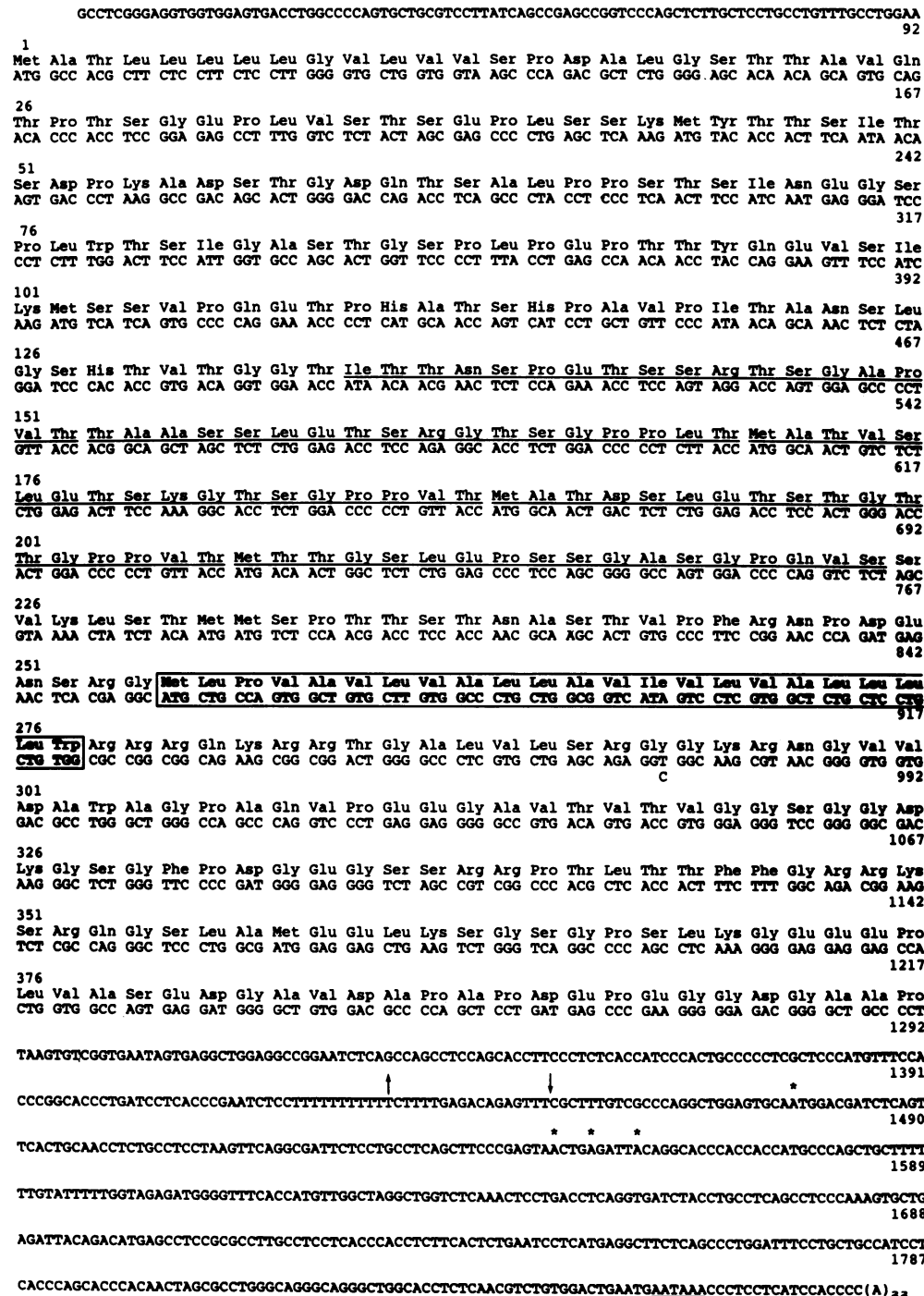


FIG. 2. Nucleotide and deduced amino acid sequence of sialophorin cDNA. The depicted sequence is that of the HPB1.9 insert. Amino acids are numbered on the left and nucleotides on the right. The transmembrane domain is boxed and emboldened. The five 18-amino acid repeats are underlined, as is the polyadenylation signal sequence. Asn-239 is the single N-linked glycosylation site. The sequenced residues of the five tryptic peptides are as follows: residues 247-253, 297-311, 339-348, 353-362, and 363-370. The only discrepancy is residue 360, identified as lysine by amino acid sequencing and glutamic acid in the deduced sequence. Asterisks indicate where adenosines in HPB1.9 are replaced by guanosines in HPB2.5. Arrows pointing away from and toward the nucleotide sequence indicate, respectively, where a thymidine is missing or added in HPB2.5 relative to HPB1.9. The 45-base antisense oligonucleotide probe 3'-TTGCCGACACCCTCGGGACCCGGCCGGGGCGGTCCACGGGCTC-5' used to screen the λ gt11 CEM cDNA library contains six mismatches to the sialophorin cDNA sequence at nucleotides 986, 1004, 1007, 1010, 1019, and 1022.

onines. A 12-residue "naked" (nonglycosylated) extracellular region is located immediately adjacent to the cell membrane junction; this region (residues 243–254), the longest extracellular nonglycosylated region identifiable, includes Ser-252, known to be nonglycosylated because it was detected by amino acid sequencing.

Evolution/Conservation of Sialophorin Structure. In the extracellular region, the consensus sequence Met-Ala-Thr-Xaa-Ser-Leu-Glu-Thr-Ser-Xaa-Gly-Thr-Ser-Gly-Pro-Pro-Val-Thr occurs five times between residues 135 and 224 (Fig. 1B), suggesting that sialophorin evolved, at least in part, by intragenic duplication. A search of the protein data base indicates that the duplicated sequence is not present in any other listed protein. Moreover, no clear match was found between the sialophorin sequence and any other protein sequence.

The counterpart protein in rat has a deduced amino acid sequence totaling 371 residues (34). It contains no N-glycosylation sites. Within the rat extracellular domain the sequence Pro-Pro-Val-Thr is repeated three times (34) in a region corresponding to the human region with the five repeats. The rat and human transmembrane regions, both 23 residues long, share 70% identity as calculated by the ALIGN program (25) and the intracellular regions, 124 and 123 residues, respectively, share 72% identity. The extracellular regions, 224 and 235 residues, respectively, share only 43% sequence identity. The lower level of sequence identity in the extracellular region might be explained as follows. Selective pressures act on "naked" polypeptides to retain the primary amino acid sequence that defines tertiary structure and function. However, on heavily glycosylated polypeptides, selective pressure might act to retain only composition—i.e., to retain serines and threonines to carry carbohydrate units and prolines and/or glycines to maintain a predominantly rod-like structure. The rat and human extracellular regions have closely related atypical compositions with serine plus threonine at 40% and 38%; proline at 11% and 12%; and O-linked carbohydrate, primarily sialic acid-bearing Gal-GalNAc, at 75 and 84 estimated units per molecule, respectively. The high degree of conservation of rat and human sialophorin probably reflects structural constraints imposed by the functional roles of the molecule.

Transient Expression of Sialophorin cDNA in COS Cells. To examine cDNA-directed expression in mammalian cells, HPB2.5 was transfected into COS monkey cells, and the sialophorin precursor, which lacks the extensive glycosylation of the mature molecule, was examined after pulse-labeling with [³⁵S]methionine. The precursor was found in transfected, but not untransfected, COS cells. It co-migrates with the sialophorin precursor of CEM cells and HPB-ALL cells (Fig. 3a), indicating that the sialophorin polypeptide is correctly synthesized in the transfected cells.†

The HPB2.5-transfected COS cells were also examined for content of mature sialophorin by immunoblotting with L10, a monoclonal antibody directed against mature sialophorin (24). L10-reactive molecules, not detected in untransfected COS cells, were detected in transfected cells (Fig. 3b, lanes 1 and 2). Whereas the L10-positive molecules of CEM and HPB-ALL cells migrate as discrete bands with apparent molecular weights of 135,000 and 115,000, respectively (24) (Fig. 3b,

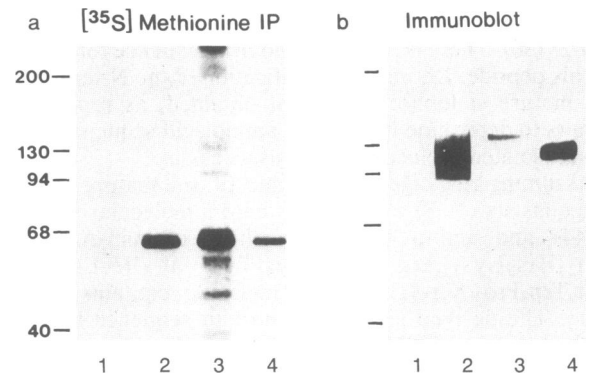


FIG. 3. Transient expression of sialophorin cDNA in COS cells. (a) Sialophorin precursor detected by [³⁵S]methionine pulse-labeling and immunoprecipitation (IP). Shown is an autoradiograph of the precipitated material of untransfected COS cells (lane 1), COS cells transfected with HPB2.5 (lane 2), HPB-ALL cells (lane 3), and CEM cells (lane 4) analyzed by NaDodSO₄/PAGE. Molecular weight marker positions are indicated ($\times 10^{-3}$). The major precipitated component was previously identified in CEM cells as the sialophorin precursor (9). (b) Lysates of cells (as identified above) immunoblotted with monoclonal antibody L10.

lanes 3 and 4), those of transfected COS cells migrate as a broad band with apparent molecular weights of 100,000–135,000. Thus, COS cells transfected with HPB2.5 synthesize the polypeptide portion of sialophorin correctly; however, the "mature" sialophorin in COS cells appears to have undergone glycosylation that is heterogeneous and different from that of sialophorin of CEM cells and HPB-ALL cells.

Chromosomal Localization of the Sialophorin Gene. Southern blot analysis (23) of genomic DNA digested with *Pst* I, *Eco*RI, *Pvu* II, and *Hind*III restriction enzymes gave single bands or simple banding patterns with sialophorin cDNA probes (data not shown), indicating a single locus. The chromosomal location of the sialophorin gene was determined by analysis of DNA from a panel of human-rodent somatic cell hybrids using a 0.3-kb probe from the 5' end of CEM1.7 (Fig. 4A). *Eco*RI-digested human DNA gave a single hybridization fragment of 22 kb (Fig. 4A, lanes 16 and 17) not detected in *Eco*RI-digested rodent DNA (Fig. 4A, lanes 14 and 15). In analysis of *Eco*RI-digested somatic cell hybrid DNA, the presence or absence of the human-specific band correlated in all instances with the presence or absence of human chromosome 16 (Fig. 4B).

CONCLUSIONS

The isolation of sialophorin cDNA clones has allowed the sequence characterization of a molecule integral to a potentially important T-cell activation pathway. Sialophorin is a highly conserved transmembrane glycoprotein both in terms of amino acid sequence of the intracellular and transmembrane regions and amino acid and carbohydrate composition of the extracellular mucin-type region. This relatively high level of evolutionary stability indicates functional importance. Sialophorin is phenotypically defective in the immunodeficiency Wiskott–Aldrich syndrome, a major manifestation of which is defective T-cell function. Since inheritance of Wiskott–Aldrich syndrome is X chromosome-linked, the localization of the human sialophorin gene to chromosome 16 excludes sialophorin as the primary genetic defect in this disease and indicates that the X chromosome product is required for normal surface expression of sialophorin. We speculate that the defect of the X chromosome product and/or the resulting sialophorin defect in Wiskott–Aldrich lymphocytes compromises their maturation, their activation by the sialophorin pathway, or their survival in the circulation.

†There is considerable disparity between the apparent molecular weight of the sialophorin precursor as estimated by electrophoretic mobility ($M_r = 62,000$, or $M_r = 58,000$ after removal of N-linked carbohydrate) (9); and the calculated molecular weight for the deduced polypeptide ($M_r = 38,444$). The discrepancy may occur because correlation of molecular weight with electrophoretic mobility (35) does not necessarily apply to polypeptides like sialophorin that have atypical amino acid composition. Alternatively, other moieties, possibly GalNAc, may be present on the precursor polypeptide.

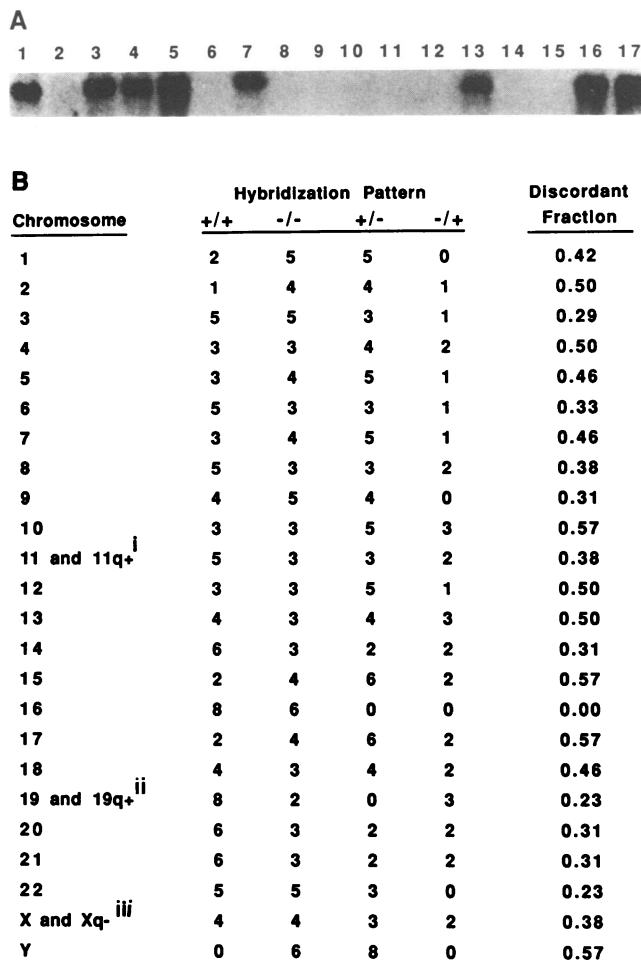


FIG. 4. (A) Southern blot analysis of *Eco*RI-digested DNA from a panel of human-rodent somatic cell hybrids. DNA (20 μ g) from a characterized somatic hybrid (lanes 1-13) (36-39), hamster (lane 14), mouse (lane 15), and human cells (lanes 16 and 17) is shown. Probe CEM-E/N (Fig. 1A) gives a single 22-kb band in DNA containing the human sialophorin gene. (B) Correlation of the presence of the human sialophorin gene with the presence of each human chromosome in the somatic cell hybrids. The hybridization patterns are as follows: +/+, hybridization signal and chromosome both present; -/-, hybridization signal and chromosome both absent; +/-, hybridization signal present but chromosome absent; -/+, hybridization signal absent with chromosome present. For calculation of discordant fractions, hybrids with a rearranged chromosome or in which the chromosome was present in <15% of metaphases were excluded. i, This hybrid clone is from a fusion of mouse A9 cells with fibroblasts from a 11/X translocation carrier (38); ii, eleven hybrid clones derived from fusions with leukocytes from two X/19 translocation carriers; iii, the X category includes hybrids with intact X and with X translocation chromosomes.

We thank Steven Burakoff, Barbara Bierer, and Catherine Wachtler for help and advice, Brian Seed, Robert Finberg, and Stuart Orkin for providing DNA libraries, and the MIT Cell Culture Center for large-scale cultures of CEM cells. This work was supported by Grants AI21163, GM37298, and HD22082 from the National Institutes of Health and by Grant 6370 from the March of Dimes Birth Defects Foundation. A.S.W. and M.C.C. are Pew Scholars in the Biomedical Sciences. A.E.D. is a recipient of an American Heart Association Established Investigator Award.

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