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Immunology

Molecular cloning of complementary DNAs encoding the heavy chain of the human 4F2 cell-surface antigen: A type II membrane glycoprotein involved in normal and neoplastic cell growth

(T-cell activation antigen/cDNA expression library)

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ABSTRACT **Complementary DNA (cDNA) clones encoding the heavy chain of the heterodimeric human membrane glycoprotein 4F2 have been isolated by immunoscreening of a λgt11 expression library. The identity of these clones has been confirmed by hybridization to RNA and DNA prepared from mouse L-cell transfectants, which were produced by whole cell gene transfer and selected for cell-surface expression of the human 4F2 heavy chain. DNA sequence analysis suggests that the 4F2 heavy-chain cDNAs encode an approximately 526-amino acid type II membrane glycoprotein, which is composed of a large C-terminal extracellular domain, a single potential transmembrane region, and a 50–81 amino acid N-terminal intracytoplasmic domain. Southern blotting experiments have shown that the 4F2 heavy-chain cDNAs are derived from a single-copy gene that has been highly conserved during mammalian evolution.**

The human 4F2 antigen is a 120–125 kDa disulfide-linked heterodimeric membrane glycoprotein, which is composed of an 80–90 kDa glycosylated heavy chain and a 38 kDa nonglycosylated light chain (1–5). The molecule was originally identified by the production of a murine monoclonal antibody (mAb4F2) raised against the human T-cell tumor line HSB-2 (1). Initial studies utilizing anti-4F2 antibodies revealed that the antigen is present on all established human tissue culture cell lines and the majority of malignant human cells (1–6). While 4F2 is not expressed at significant levels on resting B and T lymphocytes, its expression can be readily induced following lectin or alloantigenic stimulation of resting human T cells. In fact, 4F2 is the earliest known T-cell activation antigen; it first appears 4 hr following lectin stimulation of resting T cells, before the expression of interleukin 2 and transferrin receptors or the onset of DNA synthesis (7).

Although the precise function of the 4F2 molecule remains unknown, a number of recent investigations have provided intriguing data concerning the role of this molecule in the regulation of intracellular calcium concentration and the concomitant control of growth, excitability, and endocrine secretion. A role for 4F2 in cell growth and/or division has been suggested by its ubiquitous presence on rapidly dividing cells and by the finding that mAb4F2 blocks tritiated thymidine incorporation by cultures of lectin-activated T lymphocytes (1). More recently, Hashimoto and colleagues have described several murine monoclonal antibodies that recognize a 125-kDa glycoprotein with an identical tissue distribution and structure to the 4F2 antigen (5). These antibodies have been shown to efficiently inhibit the growth of four human and rat tumor cell lines *in vitro* (8).

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Several independent lines of investigation have provided evidence that 4F2 is intimately involved in the regulation of intracellular calcium concentration. First, Posillico *et al.* (9) have shown that incubation of cultured human parathyroid adenoma cells with mAb4F2 raises the concentration of intracellular calcium and results in a concomitant decrease in the basal level of parathyroid hormone secretion by these cells. In addition, Michalak *et al.* (10) have reported that preincubation of skeletal muscle or cardiac sarcolemmal vesicles with mAb4F2 inhibits 90% of the Na^+-dependent Ca^{2+} uptake by these vesicles, suggesting that 4F2 may be the Na^+/Ca^{2+} exchanger or, alternatively, may be involved in its regulation. A third role for the 4F2 molecule has been suggested by Hercend and coworkers (11), who have recently described a subset of human natural killer cells that specifically recognizes and kills target cells bearing the 4F2 antigen.

Given these previous findings, it was of interest to determine the structure of both chains of the 4F2 antigen. In this report, we describe the isolation and characterization of cDNA clones encoding the heavy chain of the human 4F2 molecule.

MATERIALS AND METHODS

Whole Cell Gene Transfer. High molecular weight DNA was prepared from the human T-cell tumor line HPB-ALL (12, 13). Whole cell gene transfer was performed into mouse Ltk^- cells using calcium phosphate-precipitated HPB-ALL and pHSV106 (14) (containing the herpes simplex virus thymidine kinase gene) DNAs (15). Approximately 9000 hypoxanthine/aminopterin/thymidine (HAT)-resistant colonies were screened by indirect immunofluorescence using mAb4F2. The brightest 1–3% of the transfectants were sterilely collected and regrown. Immunofluorescent cell sorting was repeated three times until a homogeneous population of primary 4F2-expressing cells (L4F2) was obtained. This protocol was repeated using high molecular weight DNA isolated from the primary L4F2 transfectants to produce secondary transfectants.

Preparation and Characterization of Rabbit Anti-4F2 Antiserum. The 4F2 protein was purified from membrane preparations of HPB-ALL cells by passage over a mAb4F2 immunoaffinity column and elution in 3M potassium thiocyanate (16). The immunoreactive material was reduced and purified by preparative $NaDodSO_4$/PAGE followed by electroelution (17). One rabbit was immunized by subcutaneous injection of approximately 25 μg of purified protein in complete (first immunization) or incomplete (subsequent immunizations) Freund's adjuvant every 2–3 weeks for a total of four immunizations. Serum was obtained 7 days after the fourth immunization and was tested by immunoprecipitation (18) and by immunological blotting (18).

Construction and Screening of the JY λgt11 cDNA Library. Poly(A)$^+$ RNA from the Epstein–Barr virus-transformed human B-cell line JY (19) was used to construct a λgt11 cDNA library (21). The J55 rabbit anti-4F2 heavy-chain antiserum (at a 1:1000 dilution) in conjunction with a goat anti-rabbit alkaline phosphatase-coupled second antibody (Promega Biotec, Madison, WI) was used to screen 750,000 recombinant plaques (20). Additional cDNA clones were isolated by hybridization of ^{32}P-labeled 4F2 heavy-chain cDNAs to λgt10 cDNA libraries from JY (21) and the human T-cell tumor line HPB-MLT (22).

DNA Sequencing. Appropriate restriction enzyme fragments were subcloned directly from low-melt agarose (23) into M13mp18 and M13mp19 for sequencing by the dideoxy method of Sanger *et al.* (24). All restriction enzyme sites were crossed, and all clones were sequenced on both strands.

RNA and Southern Blotting. RNA blot hybridizations were performed using Biotrans nylon filters (ICN) (15). Southern blotting was performed as described (15) except that 5% dextran sulfate (Oncor, Gaithersburg, MD) was included in the hybridization solutions.

RESULTS

Whole Cell Gene Transfer of mAb4F2 Reactivity. Mouse Ltk$^-$ cells were transfected with calcium phosphate-precipitated high molecular weight DNA from human HPB-ALL cells in conjunction with the herpes simplex virus thymidine kinase gene. HAT-resistant cells expressing the human 4F2 antigen (L4F2) were selected by three sequential rounds of indirect immunofluorescent cell sorting using mAb4F2 (Fig. 1). Similar 4F2-expressing L-cell transfectants have been previously described (25, 26). High molecular weight DNA from these primary L4F2 transfectants was used to produce secondary transfectants by an identical protocol.

Reactivity of the J55 Rabbit Anti-4F2 Antiserum. The 4F2 heavy-chain protein was purified by immunoaffinity column chromatography followed by preparative NaDodSO$_4$/PAGE and was used to raise the J55 rabbit antiserum. This heteroserum was shown to recognize the denatured 4F2 heavy chain at dilutions of 1:10,000 in immunological blotting experiments and to specifically immunoprecipitate the 4F2 protein from lysates of surface-labeled HPB-ALL cells (Fig. 2).

Identification of 4F2 Heavy-Chain cDNA Clones. The J55 antiserum was used at a dilution of 1:1000 to screen 7.5×10^5 recombinant λgt11 clones from a JY cDNA library. Seventeen positive clones were identified and purified to homogeneity by sequential immunoscreening. Four of these clones exhibited strongly positive signals on immunoscreening and were shown to cross-hybridize in Southern blotting experiments (data not shown). The remaining 13 positive clones displayed less intense signals during antibody screening and were not further characterized. Of the four strongly reactive clones, one (16A2) was 2.5 kilobases (kb) in length while the remaining three were 650 base pairs (bp) long (data not shown). The 16A2 clone and one of the three shorter clones (8B1) were chosen for further characterization. In addition, several homologous cDNAs were identified by hybridization to the radiolabeled 8B1 cDNA.

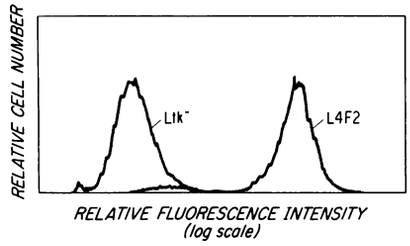

FIG. 1. Indirect immunofluorescence analysis of Ltk$^-$ and primary L4F2 transfectant cells with mAb4F2. Mouse Ltk$^-$ cells were cotransfected with high molecular weight human DNA and the herpes simplex virus thymidine kinase gene and were subjected to three sequential rounds of indirect immunofluorescent cell sorting with mAb4F2. The Ltk$^-$ recipient cells and the three-times sorted L4F2 transfectants were stained with mAb4F2 and analyzed on an EPICS V fluorescent-activated cell sorter.

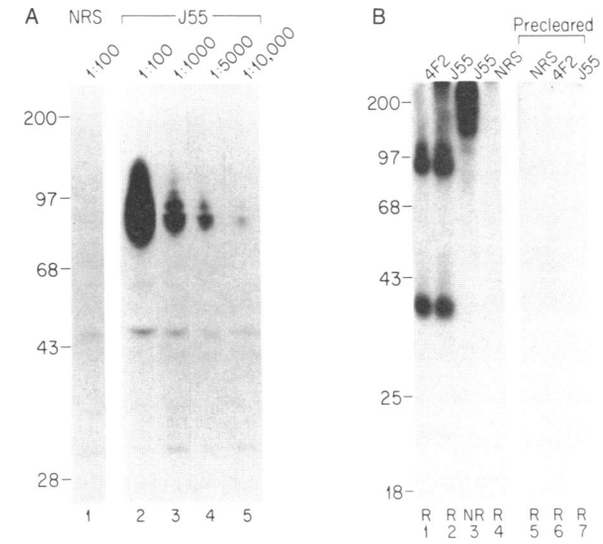

FIG. 2. Reactivity of the J55 rabbit anti-4F2 antiserum. (*A*) Immunological blot analysis of the J55 rabbit antiserum. Reduced whole cell lysates of HPB-ALL cells were subjected to immunological blot analyses using a 1:100 dilution of preimmune rabbit antiserum (NRS; lane 1) or various dilutions of the J55 anti-4F2 heavy-chain antiserum (lanes 2–5). (*B*) Immunoprecipitations of ^{125}I-surface-labeled HPB-ALL cells with preimmune rabbit antiserum (NRS; lanes 4 and 5), mAb4F2 (lanes 1 and 6), or the J55 rabbit anti-4F2 heavy-chain antiserum (lanes 2, 3, and 7). Lanes 1–4 show direct immunoprecipitations with the various antibody preparations. Lanes 5–7 show immunoprecipitations of an ^{125}I-labeled HPB-ALL lysate that was first precleared with mAb4F2 and then subjected to immunoprecipitation with the listed antibodies. Immunoprecipitations were performed under reducing (R) or nonreducing (NR) conditions.

In order to demonstrate that these clones do, in fact, encode the 4F2 heavy chain, they were hybridized to DNA (Fig. 3) and RNA (Fig. 4) prepared from primary and secondary L4F2 transfectants. Hybridization of these cDNAs to Southern blots identified a set of genomic bands from human T-cell DNA and a distinct set of hybridizing bands from mouse Ltk$^-$ cell DNA (Fig. 3*A*). The secondary L4F2 transfectants contained both the human and the mouse bands.

Hybridization of the 8B1 and 16A2 cDNAs to blot hybridizations containing RNA from human HPB-ALL cells, mouse Ltk$^-$ cells, and primary L4F2 transfectant cells (L4F2T1 and L4F2T2) showed that the transfectants express an RNA of approximately 2.1 kb, which is identical in size to that expressed by the human cells (Fig. 4). No RNA corresponding to the 8B1 cDNA clone is seen in the Ltk$^-$ lane (Fig. 4). However, upon longer exposures, a faint 2.1-kb band has been detected in the mouse Ltk$^-$ RNA (data not shown). Taken together, these data indicate that the 8B1 clone encodes the 4F2 heavy-chain gene that has been transferred to the L4F2 transfectants. They also show that there is a significant level of homology between the mouse and human genes. In fact, the 8B1 clone has recently been used to isolate murine 4F2 heavy-chain cDNAs by low-stringency hybrid-

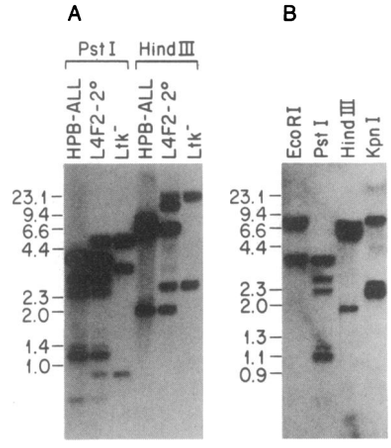

FIG. 3. Southern blot analysis of human and mouse DNAs with the 4F2 heavy-chain cDNA J1FB. (*A*) High molecular weight DNAs from the human T-cell tumor cell line HPB-ALL, the mouse Ltk⁻ recipient cells (Ltk⁻), and the 4F2-expressing secondary mouse L-cell transfectants (L4F2-2°) were digested with the appropriate restriction enzymes and subjected to Southern blot analysis. Molecular size markers are shown in kb to the left of each set of blots. (*B*) Five micrograms of HPB-ALL DNA was loaded in each lane. Low-stringency washing conditions [0.1× SSC (1× SSC = 0.015 M sodium chloride/0.015 M sodium citrate, pH 7)/0.1% $NaDodSO_4$, 50°C] were used in an attempt to detect homologous genes.

ization to a mouse cDNA library (K.G. and J.L., unpublished results).

Structure of the 4F2 Heavy-Chain cDNAs. In order to ascertain the structure of the 4F2 heavy chain, the nucleotide sequences of several 4F2 heavy-chain cDNAs were determined (Fig. 5). The salient features of the consensus 4F2 heavy-chain cDNA sequence can be summarized as follows: The full length cDNA is 1854 bp long. It contains a 1687-bp open reading frame beginning at its 5′ end. A stop codon at nucleotide 1687 is followed by a 143-bp 3′ untranslated region and a consensus polyadenylylation signal (AATAAA) at nucleotide 1830. This is followed 18 bp downstream by a poly(A) tail. The precise 5′ end of the cDNA is unclear, as the 16A2, J1FB, M3F, and J1A clones begin within 10 bp of one another at their 5′ ends.

The 16A2 cDNA clone was found to contain two anomalies, which presumably represent artifacts of the cDNA cloning procedure (Fig. 5): (*i*) The 16A2 clone is a cDNA dimer between the bona fide 4F2 heavy-chain cDNA [from nucleotide 1 to the polyadenylylation signal and poly(A) tail

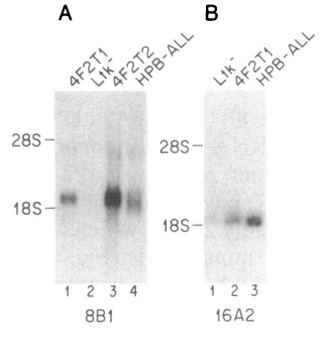

FIG. 4. Blot-hybridization analysis of human and mouse RNAs with the human 4F2 heavy-chain cDNAs 8B1 and 16A2. Ten micrograms of whole cell RNA from the mouse Ltk⁻ recipient cells (Ltk⁻), human HPB-ALL cells (HPB-ALL), and two different pools of L4F2 transfectants (L4F2T1 and L4F2T2) were subjected to blot-hybridization analysis using radiolabeled 8B1 (*A*) and 16A2 (*B*) cDNA probes.

at nucleotide 1830] and a second unrelated cDNA (nucleotides 1860–2500). This finding was consistent with the fact that the 16A2 clone was approximately 2.5 kb in length, while the size of 4F2 heavy-chain mRNA, as estimated from RNA blots, was only 2.1 kb. (*ii*) The 16A2 clone contained 10 deoxyadenosine nucleotides beginning at nucleotide 688, whereas the J1FB and M3F cDNAs contained only 8 deoxyadenosine nucleotides at the corresponding position. This alteration in the 16A2 clone produces a frame shift, which results in a stop codon at nucleotide 787 of this clone. In order to determine the correct number of deoxyadenosine nucleotides at this position, a genomic clone corresponding to this area of the cDNA was isolated from a genomic library produced from the HPB-ALL cell line (K.G., N.J., E.Q., and J.L., unpublished results). Sequence analysis of this region of the genomic clone reveals the presence of 8 deoxyadenosine nucleotides, in agreement with the sequences of the J1FB and M3F cDNA clones.

An analysis of the predicted protein structure shows that the cDNAs encode a protein of 495–526 amino acids (depending upon which AUG of the mRNA is used for the initiation of translation; for the sake of clarity all numbering will be done assuming that the first AUG is the correct site of initiation of translation) with a predicted molecular mass of approximately 58 kDa. This correlates well with previous estimates (2) of the size of the unglycosylated heavy chain (65 kDa). Hydrophilicity plots (Fig. 6) reveal that the protein contains one major, hydrophobic, potential membrane spanning domain between amino acids 82 and 101. This region is preceded by two basic residues at positions 79 and 81, a finding consistent with the hypothesis that this hydrophobic domain spans the cell membrane. A second hydrophobic domain occurs between amino acids 347 and 365. This domain could represent a second membrane spanning region. However, it is less hydrophobic and might also represent an extracellular hydrophobic internal region of the molecule. Of note, there is no 5′ hydrophobic signal sequence in the predicted protein structure. The protein contains two cysteine residues (positions 106 and 327) and four potential N-linked glycosylation sites. It is noteworthy that all of the N-linked glycosylation sites and both cysteines lie on the C-terminal side of the potential membrane spanning region. These results, when taken together with the fact that the 4F2 heavy chain is known to be disulfide-linked and N-glycosylated (1, 2), suggest that this molecule is a type II membrane protein, which is oriented with its C-terminus in the extracellular space and its 50–81 N-terminal residues in the cytoplasmic space.

The precise N-terminus of the heavy chain remains unclear. Attempts to obtain N-terminal sequence data from purified protein have, thus far, been unsuccessful, suggesting that the N-terminus may be blocked. There are four potential ATG start codons in the first 210 nucleotides of the cDNA sequence (nucleotide positions 110, 134, 179, and 203). Those at positions 110 and 203 are closely related to the consensus ATG [CCRCCATG(G)], where R = purine, as described by Kozak (28). Of note, the putative 5′ untranslated region (nucleotides 1–109) of the 4F2 cDNA is quite G+C-rich (65% overall) and contains nine CpG dinucleotides.

DISCUSSION

Several cDNA clones encoding the heavy chain of the 4F2 molecule have been isolated by immunoscreening a λgt11 expression library and characterized by DNA sequence analysis. Given the predicted protein structure of the molecule, it is intriguing to speculate both upon its possible function(s) and the mechanisms involved in regulating its expression.

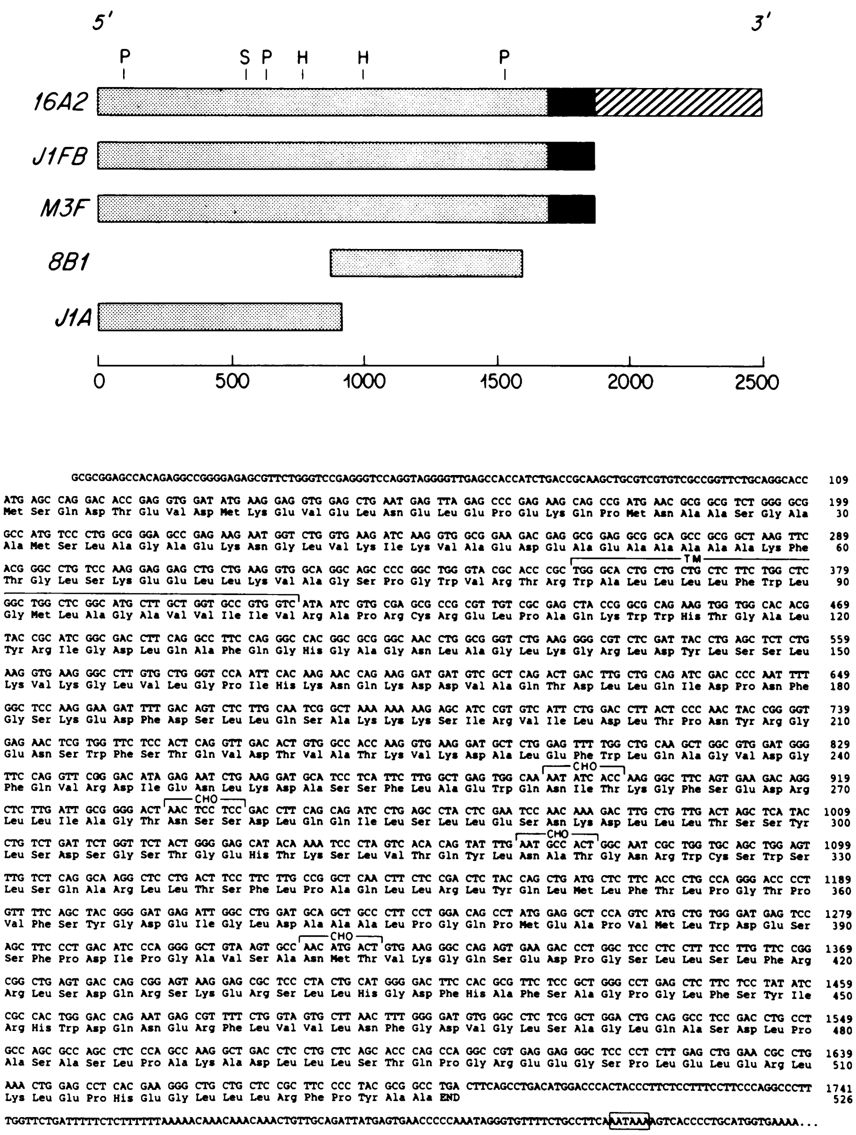

FIG. 5. Primary structure of cDNA clones encoding the 4F2 heavy chain. A schematic depiction of five cDNA clones is shown above the consensus DNA sequence of the 4F2 heavy-chain cDNAs. Stippled bars represent open reading frames. Shaded bars represent 3′ untranslated sequences. The crosshatched bar in the 16A2 clone represents artifactual 3′ sequences resulting from cDNA dimerization during the cloning procedure. The 5′ ends of the 16A2, J1FB, M3F, and J1A cDNAs begin within 10 bp of one another. Sizes in kb are shown below the schematic drawings. Restriction enzyme sites are shown on the top of the schematic drawings. P, *Pst* I; S, *Sac* I; H, *Hin*cII. Putative transmembrane (TM) and N-linked glycosylation (CHO) sites are shown above the DNA sequence. Cysteine residues are at positions 106 and 327. The consensus polyadenylylation signal (AATAAA) is boxed. The 3′ end of the sequence contains a 14-bp poly(A) tail, of which only the first 4 deoxyadenosine nucleotides are shown.

Previous reports have suggested that the 4F2 molecule may be a growth factor receptor (8) or an ion (Na^+/Ca^{2+}) exchanger (10). Searches of protein sequence databanks* with the 4F2 nucleotide and predicted amino acid sequences did not reveal any significant homology to known proteins. However, a low level of homology (9 out of 15 amino acids) was detected between the 4F2 heavy chain and a calcium binding domain present in both the bovine intestinal calcium binding protein and the S100 protein. The overall structure of the molecule differs significantly from several previously described ion channels in that it lacks multiple hydrophobic membrane spanning domains. In fact, the structure of the 4F2 heavy chain more closely resembles that of a variety of cell-surface receptors in that it is composed of a substantial extracellular domain, a single potential membrane spanning region, and a 50–81 amino acid intracytoplasmic domain. In this light, it is of interest to note that Hashimoto and colleagues have recently shown that monoclonal antibodies that are most likely directed against the 4F2 heavy chain possess potent antigrowth properties (8). Of note, the putative intracytoplasmic domain of the 4F2 heavy chain does not display significant homology with the analogous regions of previously described growth factor receptors that have been shown to possess tyrosine kinase activity (29). Experiments designed to determine whether the unregulated expression of the molecule results in the growth transformation of primary cells are necessary. In addition, a more complete understanding of the structure of the 4F2 molecule awaits the cloning and sequencing of the gene encoding the light chain of the antigen.

Southern blot analyses (Fig. 3*B*) revealed that the 4F2 heavy-chain gene probably belongs to a single gene family in that it hybridizes to three *Kpn* I, *Eco*RI, and *Hin*dIII genomic fragments. Moreover, all of these fragments are contained within a single genomic clone (K.G., N.J., E.Q., and J.L., unpublished results). These experiments also showed that this gene has been highly conserved during mammalian evolution, in that the human cDNAs detect cross-hybridizing murine genomic bands. As noted above, the 5′ untranslated sequences of the gene are quite G+C-rich and contain a greater than expected number of CpG dinucleotides, which are thought to be evolutionarily selected against because of methylation and subsequent mutation to TpG and CpA dinucleotides (30). This region of the gene resembles an *Hpa* II tiny fragment island, which has been said to be characteristic of the 5′ region of vertebrate housekeeping genes (31). These findings are also in accord with the hypothesis that the 4F2 molecule is an evolutionarily conserved housekeeping protein, which plays a central role in cell growth and/or division.

*EMBL/GenBank Genetic Sequence Database (1986) GenBank (Bolt, Beranek, and Newman Laboratories, Cambridge, MA), Tape Release 11.0; and Protein Identification Resource (1986) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 46.0.

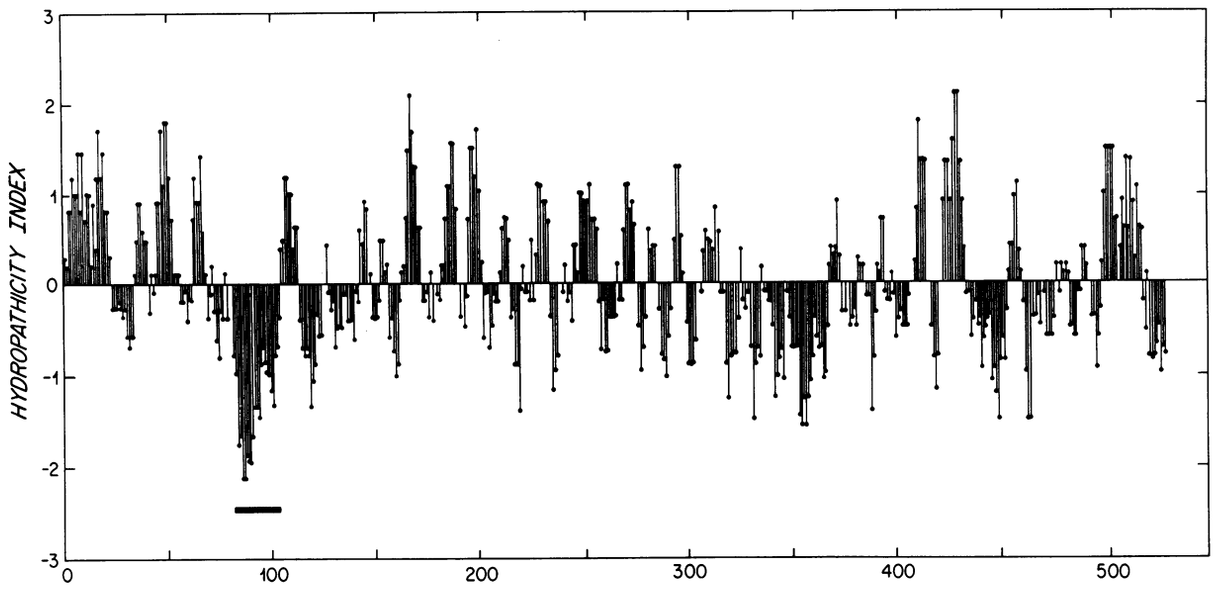

FIG. 6. Hydrophilicity (hydropathicity) plot of the predicted 4F2 heavy-chain protein. Hydrophilicity was calculated by the algorithm of Hopp and Woods (27). In this plot, increasing hydrophobicity is represented as a negative deflection on the abscissa. The putative hydrophobic transmembrane region is represented by a bar.

DNA sequence analysis suggests that the 4F2 heavy chain is a type II membrane glycoprotein with an intracytoplasmic N-terminus and an extracellular C-terminus. Thus, it is similar to the previously reported asialoglycoprotein receptor (32), the transferrin receptor (33), and the invariant chain of the HLA antigen (34). In this light, it is interesting to note that like 4F2, the transferrin receptor and HLA class II molecules are both T-lymphocyte activation antigens. In addition, the transferrin receptor, asialoglycoprotein receptor, and invariant chain may all be involved in the transport of other molecules to and from the cell surface. Perhaps 4F2 performs a similar transport function on an, as yet, unidentified ligand.

The 4F2 molecule is not expressed at significant levels on resting T lymphocytes. However, lectin or antigenic stimulation results in the rapid appearance of the molecule on the cell surface. The availability of a cDNA probe for the heavy chain of the molecule should facilitate studies designed to determine the mechanism(s) involved in the regulation of 4F2 heavy-chain gene expression following lectin activation of resting T cells. In addition, an analysis of the corresponding genomic clone should allow the identification of the sequences involved in regulating 4F2 heavy-chain gene expression. In this regard, it will be of interest to compare these 4F2 heavy-chain regulatory sequences to the analogous sequences from other known T-cell activation molecules such as interleukin 2, the interleukin 2 receptor, class II HLA, and γ-interferon.

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