Molecular cloning of the human T-lymphocyte surface CD2 (T11) antigen

(T-cell activation/DNA sequencing/cDNA expression library)

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ABSTRACT CD2 (T11, sheep erythrocyte receptor) is a surface antigen of the human T-lymphocyte lineage. cDNA clones encoding CD2 have been isolated by using the purified, denatured CD2 to raise a rat antiserum. Positive clones were recognized in a phage λ gt11 expression library prepared from the human leukemia T-cell line J6. The DNA sequence contained an open reading frame encoding 360 amino acids. The N-terminal 24 amino acids were characteristic of a signal peptide and were followed by a region that matched all 25 residues of the CD2 N terminus previously determined by amino acid sequencing. The predicted amino acid sequence is consistent with that of a transmembrane glycoprotein containing three potential N-glycosylation sites on the N-terminal side of a 26-amino acid hydrophobic segment. There is a large cytoplasmic domain of 125 amino acids that is rich in proline and in basic residues. RNA blot-hybridization analysis demonstrated hybridization only in those T cells that were positive for surface CD2 antigen. There are limited regions of sequence similarity to members of the immunoglobulin supergene family.

CD2 is a surface antigen of the human T-lymphocyte lineage that is expressed on all peripheral blood T cells. It is one of the earliest T-cell markers, being present on >95% of thymocytes. It also is found on some natural killer cells but not on B lymphocytes (1–3). Monoclonal antibodies directed against CD2 inhibit the formation of rosettes with sheep erythrocytes, indicating that CD2 is the erythrocyte receptor or is closely associated with it (1–3). There is evidence that CD2 may mediate T-cell growth, based on observations that T-cell proliferation is initiated by certain combinations of anti-CD2 monoclonal antibodies (4, 5) or by sheep erythrocytes (6). T-cell activation required the presence of two anti-CD2 monoclonal antibodies, one of which bound well to activated cells but poorly to resting cells; its epitope was revealed by the binding of the other antibody to resting cells (4, 5). There are two recent reports of candidates for a naturally occurring ligand, the action of which may be mimicked by these monoclonal antibodies. A M_r 42,000 glycoprotein on sheep erythrocytes, which has been shown to be responsible for the formation of erythrocyte rosettes with T lymphocytes (7), has also been described on sheep leucocytes (8). Another candidate is a soluble factor, released by activated helper T cells, that stimulated resting T cells to grow in a fashion similar to that induced by the anti-CD2 monoclonal antibodies (9). Activation of mature T cells through CD2 depended on the secretion of interleukin 2 (IL-2) and expression of IL-2 receptors (4), as is the case with antigen-specific activation via the T-cell antigen receptor (Ti

heterodimer–T3 antigen complex) pathway. However, activation of T-cells via CD2 and Ti–T3 appear not to be identical because phorbol esters have been shown to have opposite effects in the two systems. Thus, T-cell proliferation in the presence of a single anti-CD2 monoclonal antibody was induced by phorbol ester (10), whereas prolonged pretreatment with phorbol ester inhibited proliferation elicited by anti-T3 monoclonal antibody (11).

Anti-CD2 monoclonal antibodies are able not only to initiate T-cell proliferation but also to regulate T-cell activation mediated via the Ti–T3 complex. Thus, these antibodies strongly inhibited the proliferation of T cells induced by specific antigen (12) and anti-T3 antibody (12) as well as by the polyclonal mitogen concanavalin A (13); inhibition of proliferation was associated with lack of both IL-2 receptor expression (14) and IL-2 secretion (13). Delivery of help to B cells (13) and secretion of γ interferon (15) were also inhibited, as was T-cell cytotoxicity (16, 17).

The apparent importance of CD2 in T-cell activation provides a strong reason for cloning the gene encoding CD2. This communication describes the isolation of cDNA CD2 clones by screening an expression library with antiserum against the purified, denatured antigen. The predicted amino acid sequence of the CD2 antigen has the features of a transmembrane glycoprotein, with distant similarity to the immunoglobulin supergene family, and an extensive, proline-rich basic cytoplasmic domain.

MATERIALS AND METHODS

Purification of CD2. Purified CD2 was prepared from the human leukemia T-cell line J6 as described (36). Briefly, the antigen was separated from Nonidet P-40-solubilized microsomes by immunoabsorption chromatography with the monoclonal antibody MAR-206 (donated by A. Moretta) attached to Sepharose. The eluted antigen was finally purified by preparative NaDodSO₄/PAGE.

Polyclonal Anti-CD2 Serum. A rat antiserum was prepared by injection of 5 μ g of purified CD2 at 3-wk intervals. The material was emulsified in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for subsequent injections. All injections were given at multiple subcutaneous sites. Blood was collected 6 days after the third injection. The antiserum was tested by immunoblotting that was performed as described (18).

cDNA Library Screening. A cDNA library, constructed in the phage vector λ gt11 (19), was prepared from J6 mRNA (18). Screening was performed as described (18) with the antiserum at a dilution of 1:100. Positive clones were picked and subjected to three further rounds of screening. Library screening by DNA hybridization was performed by standard

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Abbreviations: IL-2, interleukin 2; kb, kilobase(s); V, variable; C, constant.

procedures (20) with a final wash in $0.2\times$ NaCl/Cit ($1\times$ NaCl/Cit = 0.15 M NaCl/ 0.015 M sodium citrate) containing 0.1% NaDodSO₄ for 1 hr at 68°C . DNA was labeled with ^{32}P by random hexanucleotide priming (21) to a specific activity of $1\text{--}2 \times 10^9$ cpm/ μg .

DNA Sequencing. Inserts from positive $\lambda\text{gt}11$ clones were subcloned into the plasmid vector pUC13 or into bacteriophage M13 sequencing vectors. Appropriate restriction fragments were prepared and subcloned into M13. DNA sequence analysis was performed by the dideoxy chain-termination method (22) using ^{35}S -labeled deoxycytidine 5'-[α -thio]triphosphate or [^{32}P]dCTP. Some sequence data was obtained by specific priming of M13 clones with synthetic 17'-mer oligonucleotides prepared by I. Goldsmith (Imperial Cancer Research Fund, London). The entire sequence was determined on both strands.

RNA Blotting. RNA was prepared by using Nonidet P-40 (23). Preparation of poly(A)⁺ RNA, denaturation, electrophoresis in gels containing formaldehyde, and transfer to nitrocellulose were performed as described (20). Filters were hybridized overnight in 50% (vol/vol) formamide at 42°C (20) with cDNA labeled by random priming (21). The final washes were two incubations for 15 min each at 50°C in $0.1\times$ NaCl/Cit containing 0.1% NaDodSO₄.

RESULTS

Reactivity of Anti-CD2 Antiserum. Purified, denatured CD2 was used to raise an antiserum in rats and to determine the N-terminal 26 amino acid sequence (the latter sequence is shown in Fig. 2 Lower). The rat antiserum was tested by immunoblotting of lysates of J6 cells. It revealed a broad band of $M_r \approx 50,000$, coincident with that obtained with the anti-CD2 monoclonal antibody, RFT11 (Fig. 1a, lanes 1 and 3). This band was not detected in lysates of the human lymphoblastoid B-cell line BRI 8 (Fig. 1b, lanes 2 and 4), nor was it revealed by preimmune serum (Fig. 1b). The band was detected at an antiserum dilution of 1:320 (Fig. 1b). The only other entity specifically revealed by the antiserum was a weak M_r 25,000 band (Fig. 1a, lane 1; Fig. 1b, lanes 1 and 3).

Identification of Clones in $\lambda\text{gt}11$. The antiserum revealed nine positive clones out of 5×10^6 plaques in a $\lambda\text{gt}11$ library

FIG. 1. Immunoblots developed with anti-CD2 antiserum; 2×10^6 cell equivalents were loaded per track. (a) Lysates of whole J6 (lanes 1 and 3) and BRI 8 (lanes 2 and 4) cells were developed with the rat anti-CD2 antiserum at a dilution of 1:100 (lanes 1 and 2) or with the monoclonal antibody RFT11 (provided by G. Janossy) at a concentration of $10 \mu\text{g}/\text{ml}$ (lanes 3 and 4). (b) Lysates of J6 cells were developed with the rat anti-CD2 antiserum (tracks 1, 3, and 5) and the preimmune serum (tracks 2, 4, and 6) at dilutions of 1:32 (tracks 1 and 2), 1:100 (tracks 3 and 4), and 1:320 (tracks 5 and 6). Molecular weight markers are indicated in kDa. The arrows indicate the weak M_r 25,000 band.

containing cDNA prepared from J6 cells. These appeared to be identical, with an insert size of 1.8 kilobases (kb) that detected a similar-size transcript on RNA blot hybridization (data not shown). Preparation of the β -galactosidase fusion protein from one of these clones (named 138) (Fig. 2 Upper) revealed that the cDNA encoded a polypeptide of about M_r 10,000–15,000 only—i.e., about one-third of the anticipated size of the entire polypeptide. Sequence analysis of clone 138 demonstrated an open reading frame of 335 nucleotides in frame with the β -galactosidase open reading frame. A probe of 0.75 kb containing this open reading frame (prepared by using the *Hind*III site in clone 138; see Fig. 2 Upper) was used to screen the same library by cDNA hybridization. Positive clones were identified at a frequency of approximately 1:30,000. This procedure yielded three independent clones (namely, 141A, 141B, and 254; see Fig. 2 Upper) that extended the open reading frame of clone 138 by 0.76 kb in the 5' direction. At the 3' end, clone 141A terminated with a classic polyadenylation signal (AATAAA) 0.43 kb downstream from the termination of the open reading frame, followed 13 bases later by a poly(A) tail (Fig. 2 Lower).

Features of cDNA and Protein Sequences. The total cDNA sequence, with the polyadenylation site of clone 141A, was 1.52 kb in length, with an open reading frame of 1080 nucleotides encoding a predicted polypeptide of 360 amino acids (Fig. 2 Lower). The first 24 residues consisted of a characteristic signal peptide with an initiator methionine, a very hydrophobic central region (residues –18 to –8), and small neutral residues at positions –1 and –3 (Fig. 2 Lower). There were 11 bases of 5' untranslated mRNA, with a characteristic adenine at position –3 (24). The signal peptide was followed by a portion of predicted amino acid sequence that correctly matched all 25 residues of the previously determined N-terminal sequence of the mature CD2 polypeptide (ref. 36; see Fig. 2 Lower).

The predicted entire amino acid sequence, excluding the signal peptide, encodes a polypeptide chain of M_r 38,380, incorporating three potential N-glycosylation sites in the N-terminal half (Fig. 2 Lower). There is an intensely hydrophobic region of 26 residues, characteristic of a transmembrane region, starting at position 186. This region contains a glycine triplet (positions 194–196) that would enable the molecule to bend within the membrane. On the 3' aspect of the transmembrane region, the cDNA encodes a strongly positively charged portion, with 7 basic residues in the first 9 positions (residues 212–220). The predicted C-terminal domain has an unusual composition, with 27 prolines, 23 basic residues, and 13 glutamines in the last 100 amino acids. Excluding the C-terminal 6 amino acids, this region contains no acidic residues and no residues with aromatic side chains. There is a repetitive pattern in both the nucleotide and amino acid sequences, most obviously seen at the amino acid positions 257–270, which are homologous to positions 271–284. These particular sequences incorporate continuous runs of 5 and 4 prolines, respectively, and, overall, 9 of 14 amino acids are matched.

RNA Blotting. RNA blotting experiments using the cDNA clone 141B as a probe revealed transcripts in preparations of mRNA from activated peripheral blood T cells, the helper T-cell antigen-specific clone HA1.4 (25), and J6 cells (Fig. 3). At exposures giving a strong signal from J6 mRNA (a cell line that expresses surface CD2 antigen), there was no detectable signal with mRNA from MOLT-4 or HuT 78 cells and a very weak signal in HS-B2 cells. The latter three T-cell lines were negative for surface CD2 antigen as judged by immunofluorescence. No signals were detected with the lymphoblastoid B-cell lines BRI 8 and Maja. The principal CD2 mRNA species identified by RNA blotting was approximately 1.7 kb; there was also a weak band of about 1.3 kb.

FIG. 2. (Upper) Restriction map of the CD2 antigen cDNA. The top line represents the CD2 cDNA, with the open reading frame indicated by the thick line. Restriction sites used in the sequence analysis are shown. The arrows indicate the site and orientations (5' to 3') of synthetic oligonucleotides used as primers in the sequencing program. The positions of the cDNA clones used to define the sequence of the CD2 cDNA are shown. Clone 138 extends to about 1.0 kb beyond the 3' end of the other clones, in the direction indicated by the dashed line. (Lower) Nucleotide and predicted amino acid sequence of the CD2 cDNA. Numbers above the amino acid sequence indicate amino acid positions, whereas numbers in the right hand margin show nucleotide positions. The signal peptide (SP), potential N-glycosylation sites (CHO), and transmembrane region (TM) are overlined. The N-terminal 26-amino acid sequence determined by gas-phase sequencing for the purified CD2 antigen (NTS) is also overlined; no amino acid residue was assigned to position 23 (36).

FIG. 3. RNA blot hybridization probed with CD2 cDNA. Poly(A)⁺ RNAs (5 μ g) from Maja, BRI 8, HS-B2, J6, HuT 78, and MOLT-4 cells and poly(A)⁺ RNAs (2 μ g) from the helper T-cell clone HA1.4 and from phytohemagglutinin-activated peripheral blood T cells (PBL) were loaded per track. The probe used was the insert from clone 141B that had been labeled (8.8×10^7 cpm/ μ g) by random priming (21).

Similarity to Other Proteins. The CD2 nucleotide and amino acid sequences did not show any significant similarities to any sequence in the National Biomedical Research Foundation database (K-tuple length = 3, window size = 20, gap penalty = 2). However, there were limited regions of similarity between the CD2 amino acid sequence and that of members of the immunoglobulin supergene family. Thus, alignment of the cysteine at position 122 of CD2 with the cysteine at position 23 in the variable (V) domain of the immunoglobulin κ light chain (V_{κ} ; the N-terminal cysteine of the intradomain disulfide bridge) revealed a surrounding region of substantial identity (Fig. 4a). No identity was detected with other portions of the V_{κ} domain, nor with other immunoglobulin domains apart from a weak similarity with the constant region heavy chain C_H2 domain, where the sequence Phe-Lys-Cys-Thr is found in CD2 at positions 160–163, and in the C_H2 domain surrounding the C-terminal

FIG. 4. Similarity of the CD2 amino acid sequence to that of other proteins. Shared amino acid residues are indicated by closed boxes. (a) Residues 114–143 of CD2 are aligned with residues 297–321 of the T4 antigen (26) and with residues 15–46 of the V_{κ} region of human immunoglobulin KA (27). (b) Residues 305–313 and 321–328 of CD2 are aligned with residues 197–205 and 195–202, respectively, of the T8 antigen (28) and of the rat OX-8 antigen (29).

cysteine of the intradomain disulfide bond (27). There was even stronger similarity between this region of CD2 (i.e., the extracellular domain adjacent to the transmembrane region) and the corresponding region of the human T-cell T4 antigen. Here CD2 and T4 shared 10 of 21 continuous residues (Fig. 4a). There was another region where CD2 and T4 shared 8 of 17 continuous residues (residues 167–183 and 327–343, respectively), although the significance of these matches is questionable because the cysteines in this region are not aligned. In the cytoplasmic domain of CD2, there were two short regions rich in proline and basic residues that were similar to part of the cytoplasmic domain of the human T-cell T8 antigen and its rat equivalent, the OX-8 antigen (Fig. 4b).

DISCUSSION

cDNA clones recognized in a λ gt11 library of J6 cells by reactivity with a polyclonal antiserum prepared against the purified denatured CD2 antigen have been isolated and sequenced. Several lines of evidence indicate that these clones represent the gene encoding CD2. Most important, the putative N-terminal amino acid sequence encoded by the cDNA matches all residues of the N-terminal 25 amino acid sequence determined for the purified CD2 antigen (Fig. 2 Lower). Furthermore, the size of the predicted polypeptide (M_r 38,380) and its number of putative N-glycosylation sites (3) are in reasonable agreement with the values determined by NaDodSO₄/PAGE, both for the cell surface glycoprotein (within the range M_r 50,000–56,000) and for the non-N-glycosylated polypeptide (about M_r 40,000) derived by treatment with endoglycosidase F (36). Finally, in RNA blot-hybridization experiments, mRNA transcription of the gene was coincident with cell surface expression of the antigen. Thus, mRNA was detected in peripheral blood cells, an antigen-specific helper T-cell clone, and J6, a T-cell line known to express surface CD2 but was not detected in B-cell lines nor in T-cell lines that did not express detectable CD2 antigen (Fig. 3).

All of the putative N-glycosylation sites of the predicted CD2 polypeptide are on the N-terminal aspect of the potential transmembrane region (Fig. 2 Lower), suggesting strongly that the N terminus is extracellular. On the C-terminal side of the potential transmembrane region is a basic region of the polypeptide (residues 212–220) that is characteristic of transmembrane glycoproteins and is identified in glycophorin and the heavy chain of class I major histocompatibility antigens as being on the cytoplasmic face of the plasma membrane (30). Thus CD2 appears to be a transmembrane glycoprotein with the usual orientation—that is, N terminus extracellular and C terminus intracellular.

The size of the major species of mRNA recognized by RNA blot hybridization was ≈ 1.7 kb (Fig. 3). Use of the polyadenylation site of clone 141A would yield a mRNA species consistent in size with this band. In this case, there would be a 3' untranslated region of 0.43 kb attached to an open reading frame of 1.08 kb and a 5' untranslated region of yet undetermined size. There are two possible explanations to account for the minor mRNA species of 1.3 kb that was also detected by RNA blotting. It may encode the M_r 25,000 polypeptide recognized by the anti-CD2 antiserum in immunoblots (Fig. 1). Thus, this smaller polypeptide could be a truncated form of CD2, derived from full-length CD2 mRNA by a splicing event. Alternatively, the smaller polypeptide could be a degradation product of the mature CD2 polypeptide, and the minor mRNA band may be generated by use of the polyadenylation site (AATAAA) that starts in the last codon of the open reading frame and that incorporates the termination codon.

The initial clone recognized by the anti-CD2 antiserum (clone 138) contained at its 3' end 1.0 kb of cDNA that was

not present in the other clones. The 5' 0.76 kb of clone 138 matches clone 141A exactly as far as the first deoxyadenosine residue of the poly(A) tail of the latter clone. In the first 50 nucleotides downstream from this position, clone 138 contains two stretches of the sequence CAYTG (in which Y is an unspecified pyrimidine residue), which is frequently found in genomic DNA in the region of a polyadenylation site (31). Thus, the sequence of this region is consistent with that of genomic DNA, suggesting that this clone represents an aberrant transcript that failed to employ the normally used polyadenylation site. Alternatively, the 3' 1.0 kb of clone 138 could be the result of a double-ligation event in the generation of the cDNA library, although this possibility appears unlikely in view of the failure to detect any species of mRNA, apart from the CD2 bands, in blot hybridizations of J6 cells probed with clone 138 (data not shown).

There was significant amino acid sequence similarity between the extracellular portion of CD2 adjacent to the transmembrane region and the corresponding domain of the T4 antigen (Fig. 4a). This region of T4 was not reported to be immunoglobulin-like (26) but does contain an intrachain disulfide bond between residues 305 and 347 (32). In the corresponding domain of CD2, portions of amino acid sequence around the cysteines at positions 122 and 162 resembled sequences around the N- and C-terminal cysteines of the intradomain disulfide bonds of V_κ and C_H2 domains, respectively. These homologies suggest that this region of the CD2 gene and the immunoglobulin genes are derived from a common ancestral gene. However, the predicted secondary structure of this region of CD2 does not resemble the characteristic β -pleated sheet structure of the immunoglobulin fold but is predominantly α -helical (33). Thus, CD2 retains some primary structural homology with immunoglobulin but appears to have a different secondary structure.

The cytoplasmic domain of CD2 is large both in absolute size (125 amino acid residues) and as a proportion of the total polypeptide (37%). The relatively large cytoplasmic domain is reminiscent of transmembrane proteins involved in signal transduction, such as the receptor for epidermal growth factor (34). A large cytoplasmic domain provides, *a priori*, ample opportunity for interaction with intracellular structures. The large proportion of proline residues in the C-terminal 100 amino acids probably means that this portion of the CD2 antigen assumes an open structure, with a lack of α -helices and β structure revealed by secondary structure predictions (33). The abundance of proline and basic residues in this domain is reminiscent of the N-terminal 110 residues of the capsid protein of Semliki Forest virus (35). This region of the capsid protein potentially interacts with the viral RNA and/or with the cytoplasmic face of the viral membrane incorporating the transmembrane viral glycoproteins. Based upon this model, it is possible that this portion of the CD2 antigen may have evolved to interact with nucleic acid and/or the plasma membrane's cytoplasmic face.

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