Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure

(Expansion library/surface antigen/cloning strategy/T11 antigen)

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ABSTRACT A cDNA encoding the CD2 antigen has been isolated by a highly efficient technique based on transient expression in COS cells and adherence of cells expressing surface antigen to antibody-coated dishes. COS cells expressing CD2 cDNA isolated by this method readily formed rosettes with sheep as well as human and other mammalian erythrocytes. Pretreatment of transfected COS cells with anti-CD2 antibody, or pretreatment of human erythrocytes with anti-LFA-3 antibody, abolished rosette formation.

The ability of human thymocytes and peripheral T lymphocytes to form spontaneous rosettes with sheep erythrocytes (1–3) has been a curiosity of substantial practical, but unknown physiological, significance. Several T-cell-specific monoclonal antibodies that block erythrocyte rosette formation have been developed (4–7), and a subset of these have been shown to inhibit T-cell activation (8–10) and cytolysis (9). Conversely, an antibody identified by inhibition of cytolytic function has been shown to block rosette formation (11). All of these antibodies recognize a 50-kDa surface antigen present on thymocytes and peripheral T lymphocytes, now called CD2. Some pairwise combinations of monoclonal antibodies recognizing the CD2 molecule (but not necessarily blocking rosette formation) have been shown to cause T-cell activation (12–14). Recently, purified preparations of the potent mitogen phytohemagglutinin were shown to cause calcium influx in T cells or T leukemia cells bearing CD2 but not in cells lacking CD2 antigen or pretreated with anti-CD2 antibodies (15, 16).

In this article we describe the cloning of the CD2 surface antigen by a technique based on transient expression in COS cells and physical selection of expressing cells by adhesion to antibody-coated dishes. This approach has allowed a large number of surface antigen cDNAs to be cloned in a short period of time. A major convenience of the method is the recovery of the cDNAs of interest in a form containing the necessary sequences for surface expression. Multiple rounds of screening to identify full-length or overlapping clones are thus obviated, and large quantities of antibodies are not required. The technique allows monoclonal antibodies to be used effectively, unlike screening methods based on fusion-protein expression. The use of a high-efficiency cDNA expression vector allows the antigen of interest to accumulate at high levels on the surface of transfected cells, thus circumventing the possibility that transfected genomic sequences might fail to generate sufficient levels of protein to allow efficient selection.

MATERIALS AND METHODS

cDNA Expression Vector pπH3. A COS cell expression vector was constructed from pSV (17) by inserting a synthetic transcription unit between the suppressor tRNA gene and the simian virus 40 (SV40) origin. The transcription unit consisted of a chimeric promoter composed of human cytomegalovirus AD169 immediate early enhancer sequences fused to the human immunodeficiency virus (HIV) long terminal repeat (LTR) −67 to +80 sequences. Immediately downstream from the LTR +80 sequence was inserted a polylinker containing two BstXI sites separated by a 350-base-pair (bp) "stuffer. The BstXI sites were flanked by Xba I and Xho I sites, which could also be used to excise the insert. Downstream from the polylinker were placed the SV40 small tumor (t) antigen splice and early region polyadenylation signals derived from pSV2. The nucleotide sequence of the vector is available upon request.

cDNA Library Construction. RNA was prepared from HPB-ALL T-cell leukemia cells by the guanidinium thiocyanate/CsCl method, and cDNA was synthesized from the poly(A) fraction by the method of Gubler and Hoffman (18). The cDNA was inserted into the vector using non-self-complementary BstXI adaptors, as will be described elsewhere. The ligated DNA was transformed into Escherichia coli MC1061/p3 made competent by the protocol of Mike Scott (Department of Neurology, University of California, San Francisco; personal communication). Laboratory protocols for these and subsequent steps are available from the authors.

Recovery of cDNA Clones by Panning. Bacteriological culture dishes (Falcon 1007) were prepared for panning as described (19), except that unreacted sites were blocked by overnight incubation in phosphate-buffered saline (PBS; 8.1 mM Na2HPO4/1.5 mM KH2PO4/2.7 mM KCl/137 mM NaCl, pH 7.5) containing bovine serum albumin at 1 mg/ml. Dishes were prepared in large batches and stored frozen. In the first round of screening, twenty-four 6-cm dishes of 50% confluent COS cells (20) were transduced by speroplast fusion (21). Seventy-two hours after fusion, the cells were detached by incubation in PBS/1 mM EDTA/0.02% NaN3 at 37°C for 30 min. The detached cells were pooled, centrifuged, and resuspended in cold PBS/10 mM EDTA/5% fetal bovine serum (FBS) containing monoclonal antibodies, usually as ascites at 1:1000 dilution, but also as commercial reagents at the concentrations suggested by the manufacturers. After 1 hr on ice, the cells were diluted 1:1 with PBS/EDTA/Na2 and layered on 10 ml of PBS/EDTA/Na2 containing 2% Ficoll 400. After centrifugation (400 × g, 5 min), the supernatant was carefully aspirated, the pellet was resuspended in a small amount of PBS/EDTA/5% FBS, and the cells were distributed into 12 panning plates, each containing 3 ml of PBS/EDTA/5% FBS. After the cells were allowed to attach for 2 hr at room temperature, the plates were washed three times gently with PBS/EDTA/5% FBS. Four hundred microliters of 10 mM EDTA/0.6% NaDodSO4 was added per dish, and after 5 min the lyses were collected and treated as described (22). DNA from the first round of panning was transformed into MC1061/p3, and the resulting colonies were pooled, amplified by growth in liquid culture, and subjected
to spheroplast fusion with COS cells in six 6-cm dishes (second round). Panning with a mixture of monoclonal antibodies and rescue were carried out as before. In the third round, the transfected cells were divided into portions, and each portion was treated with antibody or antibodies specific for a particular surface antigen. Panning and transformation of the different portions gave colonies from which plasmid DNA was isolated by the alkaline miniprep method (26). The DNA from 0.5 ml of culture was then used to transfect each 6-cm COS cell culture for immunofluorescence analysis. 

COS Cell Transfection for Immunofluorescence. COS cells at 50% confluence in 6-cm dishes were transfected in 1.5 ml of Dulbecco's or Iscove's modified Eagle's medium (DMEM or IMDM, from Gibco) containing 10% NuSerum (Collaborative Research, Waltham, MA). 400 μg of DEAE-dextran per ml, 100 μM chloroquine diphosphate, and purified DNA at 1 μg/ml or miniprep DNA as above. After 4 h at 37°C (or earlier if necessary) the transfection mixture was removed and the cells were treated with 10% dimethyl sulfoxide in PBS for 2 min (23). Cells were then returned to DMEM/10% calf serum for 48–72 hr to allow expression.

Immunoprecipitation. T cells were radiolabeled by the lactoperoxidase treatment, lysed, and immunoprecipitated (24), using commercially available goat anti-mouse IgG agarose beads (Cooper Biomedical, Malvern, PA). COS cells for immunoprecipitation were transfected by the DEAE-dextran method and trypsinized and passed without dilution into new plates 24 hr after transfection. Thirty-six hours later, cells were detached by exposure to PBS/EDTA as above, centrifuged, and labeled by the lactoperoxidase method. A cleared lysate was prepared as for the T-cell immunoprecipitations, except that the lysis buffer contained 1 mM phenylmethylsulfonyl fluoride, and incubation with the primary antibody was carried out for only 2 hr at 4°C. Eluted samples were fractionated by electrophoresis in discontinuous 11.25% polyacrylamide gels (25).

RNA and DNA Blot Analyses. RNA blot analysis was carried out essentially as described (26), except that dimethyl sulfoxide was omitted from the loading buffer, denaturation was at 70°C for 5 min, and the gel contained 0.6% formaldehyde rather than 6%. The gel was stained in two volumes of water containing ethidium bromide (1 μg/ml), photographed, and transferred to nylon (GeneScreen, DuPont) in the staining liquor. The transferred RNA was irradiated by exposure to a germicidal lamp through SaranWrap (27) for 5 min at a flux (at 254 nm) of 0.22 mW/cm². DNA blot analysis was carried out by alkaline transfer to nylon (GeneScreenPlus, DuPont) (28). Hybridization probes were prepared by the method of Hu and Messing (29), and blots were prehybridized in Na2SSO4/phosphate buffer (27) containing 10 DNA microgram equivalents of M13 mp19 phage.

Erythrocyte Rosetting. Erythrocytes were prepared from whole blood by three centrifugations in PBS. COS cells were transfected in 6-cm dishes with CD2 or other surface antigen expression clones by the DEAE method. Forty-eight to seventy-two hours after transfection, the medium was aspirated and 2 ml of PBS/5% FBS/NaCl was added to each plate, followed by 0.4 ml of the appropriate erythrocyte samples as 20% suspensions in PBS. After 1 hr at room temperature, the nonadherent erythrocytes were gently washed off, and the plates were examined.

RESULTS AND DISCUSSION

Isolation of a cDNA Encoding CD2 Antigen Determinants. A cDNA library of ~3 × 10⁵ recombinants was prepared from HPB-ALL cell RNA in the expression vector pH3 and introduced into COS cells by spheroplast fusion. Three days later, the cells were detached with EDTA and treated with a pool of monoclonal antibodies, including three (OKT11, anti-Leu-5b, and Coulter anti-T11) directed against CD2 determinants. The antibody-treated cells were distributed into dishes coated with an affinity-purified sheep anti-mouse IgG antibody, allowed to attach, and separated from the nonadherent cells by gentle washing. This method of enrichment is known in the immunological literature (19) as “panning.”

Episomal DNA was collected from the adherent cells by the Hirt procedure (22) and transformed into E. coli. The resulting colonies were pooled, fused into COS cells, and subjected to a second round of panning with the pool of monoclonal antibodies as before. In the third round, a portion of the detached cells was treated with only the three monoclonal antibodies specific for CD2, and a Hirt supernatant was again generated and transformed into E. coli. DNA was prepared from eight of the resulting colonies and transfected into COS cells. After 3 days, surface expression of the CD2 antigen was detected by indirect immunofluorescence in six of eight transfected cultures. Restriction enzyme digestion of the corresponding plasmid DNAs revealed a 1.5-kilobase (kb) insert in all six isolates.

CD2 cDNA Directs the Expression of Multiple CD2 Epitopes. After transfection of the cDNA into COS cells, indirect immunofluorescence study showed that an additional panel of antibodies provided by the Third International Workshop on Leukocyte Differentiation Antigens showed that all of the antibodies provided gave positive reactions, with the exception of one sample, which also failed to react with phytohemagglutinin-activated T lymphocytes. Among the 17 antibodies tested were at least eight groups, distinguishable by their differing patterns of reactivity with lymphocytes of various primate species (30).

cDNA Sequence Analysis. The CD2 cDNA insert was subcloned into M13 mp19 (31) in both orientations, and the sequence was determined by the dideoxynucleotide method (32). The predicted amino acid sequence of 351 residues (Fig. 1) evokes a typical integral membrane protein with a single membrane-spanning hydrophobic anchor terminating in a rather large intracytoplasmic domain. Comparison of the N-terminal amino acid sequence with the matrix of signal-sequence residue frequencies constructed by von Heijne (33) suggests that the mature CD2 peptide is formed by cleavage of a precursor peptide between the 19th (serine) and 20th (lysine) residues. The membrane-spanning domain comprises 26 uncharged residues of predominantly hydrophobic character, and the cytoplasmic domain is distinctive both for its large size and its abundance of proline. Comparison of the amino acid sequence with the National Biomedical Research Foundation data base* revealed no substantive homologies with other proteins.

While this manuscript was in review, the isolation of a CD2 cDNA clone was reported by Crompton and coworkers (34). Our sequence differs from theirs at three locations: by an adenine for a cytosine in the third position of codon 247, giving glutamine instead of histidine; by an extra cytosine in codon 319, shifting the resulting reading frame; and by a thymine for a cytosine at position 1341 in the 3' noncoding region.

Immunoprecipitation of CD2 Antigen Expressed by Transfected Cells. COS cells were transfected with the CD2 expression plasmid and surface-labeled with 125I by the lactoperoxidase method 60 hr posttransfection. Immunoprecipitation and electrophoresis showed a prominent band of material precipitated from transfected COS cells by the anti-CD2 antibody, but not by the control (Fig. 2). The calculated mean molecular mass of the COS cell material was

51 kDa, compared to a mean molecular mass of 54 kDa for the T-lymphoblast and T-cell-line material; the antigen from HPB-ALL cells was found to have a molecular mass of ~61 kDa. The observed differences in size can be attributed to differing patterns of glycosylation in the different cell types; similar patterns have been observed in studies of other antigens isolated by this method (unpublished data). A minor band at apparent molecular mass 38 kDa was present in material immunoprecipitated from COS cells but not from T cells or HPB-ALL cells. The size of this species agrees within experimental error with the predicted molecular mass of the mature unglycosylated peptide, 37.3 kDa. An additional faint band of ~28 kDa, present in both COS-cell and T-cell immunoprecipitates (Fig. 2), is occasionally seen in control precipitations and is probably not CD2-related.

**COS Cells Expressing CD2 Form Rosettes with Erythrocytes.** COS cells transfected with the CD2 expression clone were treated for 1 hr with purified MT910 (IgG1,κ) anti-CD2 antibody (35) at a concentration of 1 μg/ml or with purified MB40.5 (IgG1,κ) antibody at the same concentration. MB40.5 recognizes a monomorphic HLA-ABC determinant (36) and crossreacts with African green monkey histocompatibility antigens (data not shown); it was chosen because it represents an isotype-matched antibody recognizing a surface antigen of approximately the same abundance as the CD2 antigen expressed by transfected cells. Both human and sheep erythrocyte rosettes were observed in the presence of MB40.5, but not of MT910 (Fig. 3 and data not shown). Rosette inhibition was also observed with OKT11 antibody, and not with various other control antibodies (data not shown).

In addition to sheep erythrocytes, human T cells are known to form rosettes with horse, pig, dog, goat, and rabbit erythrocytes but not mouse or rat erythrocytes (37-39). Autorosettes between human erythrocytes and human thymocytes have also been reported (40). COS cells transfected with the CD2-expression clone were treated with either MT910 or with the control antibody MB40.5 and exposed to erythrocytes from the species above. Rosettes were observed with horse, pig, dog, goat, sheep, rabbit, and human erythrocytes, but not with mouse or rat erythrocytes (data not shown). Rosette formation was blocked in all cases by pretreatment of transfected COS cells with MT910, but not MB40.5. Mouse erythrocytes showed weak spontaneous binding to the culture dish as well as to MT910- and MB40.5-pretreated cells, whereas rat erythrocytes showed no detectable binding.

**Binding of Human Erythrocytes Is Blocked by Anti-LFA-3 Antibody.** Recently, Shaw et al. (41) showed that both anti-CD2 and anti-LFA-3 antibodies inhibit conjugate formation by cytolytic T-cell clones and that the effects of blocking with anti-CD2 antibody are not potentiated by addition of anti-LFA-3 antibody, or vice versa. Based on these data, and earlier data showing that the site of action of LFA-3 antibody is the target cell (11), they proposed that LFA-3 and CD2 are involved in a common pathway for conjugate formation, possibly by direct interaction. To test this,

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**Fig. 1.** Nucleotide sequence of the CD2 cDNA insert. Nucleotide numbering is given in parentheses at right, and amino acid numbering, at center and left. Locations of the potential sites for addition of asparagine-linked carbohydrate (CHO) are shown, as well as the predicted transmembrane (TM) sequence. The amino acid number is obtained from the projected cleavage site of the secretory-signal sequence. The presumed polyadenylation signal for the 1.65-kb transcript and a possible polyadenylation signal for the 1.3-kb transcript are at positions 1590 and 1086, respectively.

**Fig. 2.** Immunoprecipitation of CD2 antigen synthesized by T cells and transfected COS cells. Cell surface proteins were labeled with 125I, immunoprecipitated, and electrophoresed in 11.25% acrylamide gel under reducing conditions. Molecular masses of standards (lane M) are given in kDa. Lanes 1-4: CD2 antigen from transfected COS cells (lane 1), from phytohemagglutinin-activated T blasts (lane 2), from HPB-ALL cells (lane 3), and from a long-term T-cell line (lane 4). Lane 5: immunoprecipitate of CD2-transfected COS cells obtained with OKT4 antibody.
transfected cells were exposed to human erythrocytes pretreated for 2 hr either with anti-LFA-3 (IgG1,κ) antibody as ascites at 1:1000 dilution or with a 10-μg/ml concentration of each of four isotype-matched nonagglutinating antibodies directed against human erythrocyte antigens as prevalent, or more so, than LFA-3: G10/B11 and D10, anti-K14 antigen; D6, anti-Wrα antigen; and F7/B9, anti-κ antigen (42). The erythrocytes were washed free of excess anti-LFA-3 antibody but were allowed to form rosettes in the presence of the control antibodies to guard against possible loss of antibody blocking power by desorption. Rosette formation was observed in the presence of all four control antibodies, but not with erythrocytes pretreated with anti-LFA-3 (Fig. 3 and data not shown). These results directly demonstrate that the adhesion reaction blocked by LFA-3 antibody is mediated by the CD2 antigen.

**COS Cells Expressing Other T-Cell Antigens Do Not Form Rosettes.** A number of clones were isolated by the same expression technique used to clone CD2 and characterized to varying degrees by antibody reactivity, nucleic acid restriction and sequence analyses, and immunoprecipitation. Representative clones were transfected into COS cells and analyzed for the ability to sustain rosette formation. Neither CD1a, CD1b, CD1c, CD4, CD5, CD6, CD7, CD8, nor CD28 (Tp44) clones were effective in forming rosettes with human erythrocytes.

**RNA Blot Analysis.** Equal amounts of total RNA prepared from cell types expressing or lacking CD2 antigen were electrophoresed in denaturing agarose gels and transferred to nylon. Hybridization of the transferred RNA with an antisense probe prepared from an M13 clone containing a CD2 cDNA insert revealed the presence of prominent 1.65- and 1.3-kb transcripts present in RNA derived from thymocyte, activated-T-cell, and senescent-T-cell populations (Fig. 4). Lesser amounts were found in RNA extracted from the cDNA donor line HPB-ALL, and less still from MOLT-4; barely detectable levels were recorded in RNA from the HSB-2 line. No reactivity was observed with RNA from the cell lines Namalwa (Burkitt lymphoma), U937 (histiocytic leukemia), HuT-78 (adult T-cell leukemia), PEER (T-cell leukemia), or Jurkat subline J3R7 (T-cell leukemia). The pattern of reactivity conformed well with the known or measured pattern of expression of CD2 antigen, which was absent or undetectable on the Namalwa, U937, HuT-78, J3R7, PEER, and HSB-2 cell lines, weakly present on MOLT-4, more strongly present on HPB-ALL, and most strongly present on activated T cells. Thymocytes are also known to express high levels of CD2 antigen.

Examination of the sequence of the cDNA clone suggested that the 1.3-kb RNA might arise by formation of an alternative 3' end distal to the canonical polyadenylation signal AATAAA at position 1085 in the cDNA sequence. To test this, RNA from HPB-ALL and activated T cells was subjected to electrophoresis followed by blot hybridization analysis either with a complete cDNA probe or with a probe derived from the 3' portion of the cDNA distal to nucleotide 1131. The latter probe reacted only with the 1.65-kb species, whereas the former showed the same reactivity pattern observed in Fig. 4 (not shown). This result is consistent with the suggested origin of the 1.3-kb transcript but does not unequivocally establish its structure.

In both activated- and senescent-T-cell RNA preparations,
a weakly hybridizing transcript of ≈0.75 kb was detected (Fig. 4). The origin of this RNA is unknown.

The CD2 Gene Is Not Rearranged. Blot hybridization analysis of genomic DNA from placenta, peripheral blood lymphocytes, T cells, HeLa cells, or the tumor lines used in the RNA analysis above showed identical BamHI-digest patterns (Fig. 5), indicating that rearrangement is not involved in the normal expression of the CD2 gene during development. Similarly, no gross genomic alteration underlies the failure of the examined T-cell tumor lines to express CD2 antigen. Restriction analysis of total genomic DNA with a number of other enzymes, as well as preliminary results with an incomplete collection of λ phage recombinants bearing CD2 sequences, shows that the gene is divided into at least four exons (data not shown).

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Fig. 5. Genomic DNA blot analysis. Twenty micrograms of each genomic DNA digested with BamHI was electrophoresed in 0.8% agarose, blotted, and hybridized to an M13-derived CD2 cDNA probe. DNA samples were prepared from the following: lane 1, HuT-78 cells; lane 2, PEER cells; lane 3, MOLT-4 cells; lane 4, HS2-2 cells; lane 5, placenta; lane 6, Jurkat J3R7 cells; lane 7, total peripheral blood lymphocytes; lane 8, HPB-ALL cells; lane 9, T blasts; lane 10, HeLa cells; and lane 11, Namalwa cells. Sizes (kb) of DNA fragments that hybridized with the probe are given at right.