

DISSECTION OF THE HUMAN CD2  
INTRACELLULAR DOMAIN  
Identification of a Segment Required for Signal  
Transduction and Interleukin 2 Production

By HSIU-CHING CHANG,\*<sup>§</sup> PHILIPPE MOINGEON,\*<sup>§</sup>  
PETER LOPEZ,<sup>‡</sup> HELEN KRASNOW,\*  
CHRISTOPHER STEBBINS,\* AND ELLIS L. REINHERZ\*<sup>¶</sup>

*From the \*Laboratory of Immunobiology and <sup>‡</sup>Flow Cytometry Facility, Dana-Farber Cancer  
Institute and Departments of <sup>§</sup>Pathology and <sup>¶</sup>Medicine, Harvard Medical School,  
Boston, Massachusetts 02115*

The 50-kD CD2 (T11) molecule, originally defined as the sheep erythrocyte receptor, plays an important role in T lymphocyte activation as well as in facilitating adhesion between T lymphocytes and their cognate partners (1-5). Perturbation of the extracellular domain of CD2 by its ligand, LFA-3, or certain anti-CD2 mAbs provides signals that synergize to augment TCR-mediated stimulation (6, 7) and initiates a rapid turnover in polyphosphoinositide accompanied by an increase in cytosolic free calcium concentration (8, 9). In addition, this same combination of anti-CD2 antibodies (with or without LFA-3) induces lymphokine production from, and clonal expansion of, resting T lymphocytes (10).

Recently, the primary structures of human and murine CD2 were deduced from cDNA and genomic cloning (11-17). The predicted structure in both species is a type I integral membrane protein. In man, the mature protein consists of a hydrophilic 185 amino acid extracellular segment, a hydrophobic 25 amino acid transmembrane segment and a 117 amino acid cytoplasmic domain. The highest degree of homology between human and murine species is found in the cytoplasmic domain (59% at the amino acid level) and in both species the cytoplasmic domain is unique with respect to its multiple proline (21%) and basic residues and by secondary structural predictions, suggesting that it has an extended nonglobular conformation. Thus, unusual but conserved features among species including man, mouse, and rat suggest an important role for the CD2 segment in signal transduction (11, 12, 18). For this reason, we have performed a series of deletion mutation studies on the CD2 cytoplasmic domain in the present report.

### Materials and Methods

*Construction of Truncated and Mutated Human CD2 Molecules and Transfection into a Murine T Cell Hybridoma.* To construct truncated CD2 molecules, the human CD2 cDNA, PB2 (11),

This work was supported by National Institutes of Health grant RO1 AI-21226. H. Chang was supported by a fellowship from the Cancer Research Institute. P. Moingeon is a recipient of a fellowship from the Philippe Foundation and Fondation pour la Recherche Medicale (France).

was digested with restriction enzymes Hph I, Ban II, Fok I, Stu I, and Ava I, respectively, and blunted with T4 DNA polymerase. The Hph I and Ban II digests were ligated to the linker 5'-CTAAGGATCCTTAG-3', while the Fok I, Stu I, and Ava I digests were ligated to the linker 5'-TAAGGATCCTTA-3' to regenerate the last amino acid, introduce a termination codon, and provide a Bam HI restriction recognition site. Subsequently, the DNAs were inserted into the Bam HI site of DOL vector (kindly provided by Dr. Thomas Roberts, Dana-Farber Cancer Institute). The substitution mutants were generated by oligonucleotide-directed in vitro mutagenesis as previously described (19). The full-length human cDNA PB2 insert was subcloned into BamHI site of M13 mp18. The synthetic oligonucleotides used for mutagenesis were 5'-CAGGCACCTAGTGATGAGCCCCCGCCTCCT-3' for CD2 M271-2, which changes the wild-type sequence CATCGT (His-Arg) into GATGAG (Asp-Glu), and 5'-CCGCCTCCTGGAGATGAGGTTTCAGCACCAG-3' for CD2 M278-9, which changes the wild-type sequence CACCGT (His-Arg) into GATGAG (Asp-Glu). The Bam HI fragment of the replicative form of M13 mutant DNAs was subcloned into expressing vector DOL. The plasmids containing full-length or modified CD2 cDNAs were isolated and sequenced around the modified region by double-stranded sequencing before transfection by  $\text{Ca}^{2+}$  precipitation into  $\psi$ -2, a helper-free retrovirus packaging cell line. Both transiently expressed and permanent viral stocks were used to infect the murine T cell hybridoma, 3DO54.8 (kindly provided by P. Marrack, National Jewish Hospital) (20) in the presence of 8  $\mu\text{g}/\text{ml}$  polybrene (Aldrich Chemical Co., Milwaukee, WI). Procedures for the growth of  $\psi$ -2 cells, transfection of cells, harvest of virus, and infection of cells were performed as described (21). Selection was initiated 48 h after infection using 0.4 mg/ml G418 (Geneticin; Gibco, Grand Island, NY), and wells containing single colonies were expanded. The G418 resistant clones were screened by indirect immunofluorescence as described in the legend to Fig. 2, and positive clones were further sorted on the Epics V cell sorter. Cells were maintained in RPMI 1640 (Gibco Laboratories) medium supplemented with 10% heat-inactivated FCS (Flow Laboratories, McLean, VA), 50  $\mu\text{M}$  2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin (Whittaker, M. A. Bioproducts, Walkersville, MD) and 0.4 mg/ml G418.

**Flow Cytometric Analysis.** For cytometric analysis, ascites were used at a 1:200 dilution and  $10^6$  cell incubated for 30 min at 4°C. After washing in RPMI 1640 with 2% FCS, bound antibodies were detected using a 1:40 dilution of fluorescein-coupled goat anti-mouse IgG as a second antibody (Meloy, Springfield, VA). 10,000 cells were analyzed per sample on an Epics V cell sorter. Histograms represent the number of cells (ordinate) vs.  $\log_{10}$  fluorescence intensity (abscissa).

**Immunoprecipitation of CD2 Molecules.**  $10$ – $20 \times 10^6$  cells were surface labeled with 1 mCi  $^{125}\text{I}$  (IMS 30; Amersham Corp., Arlington Heights, IL) for 15 min at room temperature using the lactoperoxidase method (11). Cell lysates were prepared in RIPA buffer containing 0.15 M NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM NaF, 1 mM PMSF, and protease inhibitors. Cell lysates were precleared once with a formalin-fixed *Staphylococcus aureus* suspension, then with Affigel protein A beads (Pharmacia Fine Chemicals, Piscataway, NJ) coupled to an irrelevant antibody (anti-CD8; 21Thy2D3) before overnight incubation at 4°C with the anti-CD2 (3T4-8B5) antibody coupled to beads. Immunoprecipitates were extensively washed with RIPA buffer and run on a 10% SDS-PAGE after treatment with 5% 2-ME. The gel was dried and the autoradiograph was exposed for 2 wk at  $-70^\circ\text{C}$  with intensifying screens.

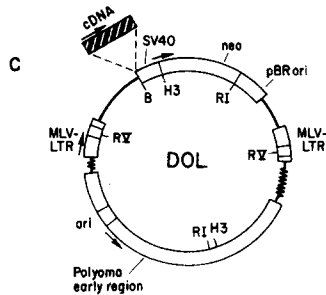
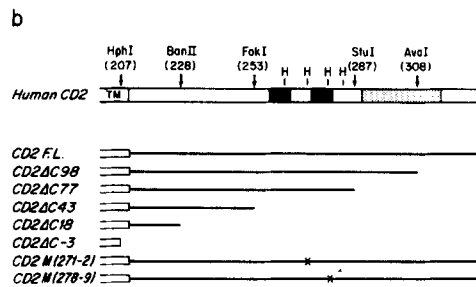
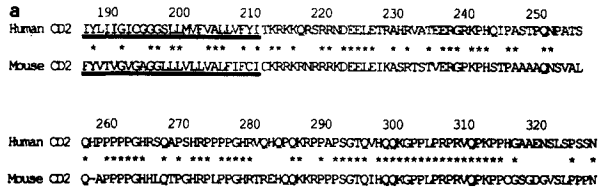
**Measurement of Cytosolic  $\text{Ca}^{2+}$  by Indo-1 Fluorescence.** Cytosolic  $\text{Ca}^{2+}$  concentrations were determined according to Gryniewicz et al. (22). Briefly,  $2 \times 10^6$  cells were loaded for 45 min at 37°C with 2  $\mu\text{g}/\text{ml}$  of the acetylmethyl ester of indo-1 (Molecular Probes, Junction City, OR) in 200  $\mu\text{l}$  of RPMI 1640 plus 2% FCS. Cells were diluted 10-fold before analysis on an Epics V cell sorter. Upon  $\text{Ca}^{2+}$  binding, indo-1 exhibits changes in fluorescein emission wavelengths from 480 to 410 nm (22). The ratio of 410/480 nm indo-1 fluorescence was recorded vs. real time and expressed in arbitrary units. One arbitrary unit represents  $\sim 200$  nM  $[\text{Ca}^{2+}]$ . For each determination, the baseline was assayed by recording indo-1-loaded cells for 1 min. Anti-T11<sub>2</sub> (1old24C1) and anti-T11<sub>3</sub> (1mono2A6) ascites were added at a 1:100 final dilution. The  $\text{Ca}^{2+}$  ionophore A23187 (Sigma Chemical Co., St. Louis, MO) was added at a 1  $\mu\text{g}/\text{ml}$  final concentration.

**Modulation of CD3 Molecules.** To modulate the murine CD3 molecule from the surface CD2 FL, cells were incubated overnight with the 145 2C11-purified antibody (20  $\mu\text{g/ml}$ ) at 37°C. Cells were washed twice and an aliquot of the cell was used to evaluate the effect of modulation on murine CD3 and human CD2 expression using a standard indirect immunofluorescence assay (with the 145 2C11 and 3T4-8B5 mAbs, respectively). Modulated cells were loaded with indo-1 and cytosolic  $[\text{Ca}^{2+}]_i$  was followed upon various stimuli. Under such conditions, CD2 FL unmodulated cells or cells modulated with the anti-human CD3 mAb (Leu4) showed a  $[\text{Ca}^{2+}]_i$  comparable to Fig. 3 *a* (not shown) when triggered via CD2.

**Proliferation of CTLL-20 Cells.** For quantitation of IL-2 production,  $10^5$  cells/well were incubated in 96-well round-bottomed plates for 24 h in the presence of either ovalbumin (1 mg/ml final concentration) plus  $10^5$  A20-11 B lymphoma cells or anti-T11<sub>2</sub> + anti-T11<sub>3</sub> (ascites 1:100) or culture medium. Incubation with the nonstimulatory combination of anti-T11<sub>1</sub> + anti-T11<sub>2</sub> antibodies did not induce any detectable IL-2 production, while stimulation with anti-T11<sub>2</sub> + anti-T11<sub>3</sub> antibodies resulted in clear IL-2 secretion by CD2 FL cells. Because addition of PMA (Sigma Chemical Co.) was found to induce a substantial increase in lymphokine production, 5 ng/ml final concentration of PMA was added to all experimental samples including the media control. Subsequently, supernatants were harvested and titrated in triplicate for their ability to support the growth of 10,000 CTLL-20 cells. Cultures were pulsed after 24 h incubation with 1  $\mu\text{Ci}$  [<sup>3</sup>H]thymidine per well and harvested after an additional overnight incubation at 37°C over glass fiber filters on a Mash apparatus. Filters were dried and counted after addition of scintillation fluid on a  $\beta$  counter. Results are expressed as mean of triplicate determinations of cpm of [<sup>3</sup>H]thymidine incorporated. Standard deviations were generally <5–10% and results are representative of five independent experiments.

## Results and Discussion

To precisely characterize the functional and structural relationship of the cytoplasmic domain of the human CD2 molecule (Fig. 1 *a*), a series of deletion mutation of the CD2 cDNA were produced, as shown in Fig. 1 *b*, encoding 98, 77, 43, 18, and -3 amino acids out of the 117 predicted cytoplasmic CD2 amino acid residues. Full-length cDNAs as well as modified cDNAs were obtained as described in Materials and Methods and inserted into the retrovirus expression vector DOL (Fig. 1 *c*) under the control of the MLV LTR promoter (24) and defective viruses were generated (21). The murine T cell hybridoma 3DO54.8 cell line specific for ovalbumin in the context of the H-2 (I-A<sup>d</sup>) molecule (20) and lacking the human CD2 was then infected with these defective retroviruses. G418-resistant clones were selected in the presence of 0.4 mg/ml G418 and analyzed for surface CD2 expression using an indirect immunofluorescence assay with anti-T11<sub>1</sub> and anti-T11<sub>2</sub> antibodies on an Epics V cell sorter. Multiple clones corresponding to each type of truncation and expressing clearly detectable levels of surface CD2 were selected for further characterization. Clones used in subsequent functional studies were designated based on the nature of their CD2 cDNA retroviral insert: CD2 FL resulted from retroviral infection with the full-length CD2 cDNA, while CD2  $\Delta\text{C}98$ , CD2  $\Delta\text{C}77$ , CD2  $\Delta\text{C}43$ , CD2  $\Delta\text{C}18$ , and CD2  $\Delta\text{C}-3$  resulted from infection with retroviruses containing the entire extracellular and transmembrane segment of CD2 but only 98 or fewer of the 117 cytoplasmic residues. A representative pattern of reactivity with the anti-CD2 mAb is shown in Fig. 2 *a*. All of these transfectants express comparable levels of CD2 ( $\sim 5$ – $10,000$  copies/cell) except CD2  $\Delta\text{C}18$ , which expresses on the order of 50% the copy number. The CD2  $\Delta\text{C}-3$  transfectant expressed levels of CD2 comparable to CD2  $\Delta\text{C}18$ . However, the surface expression of CD2 on  $\Delta\text{C}-3$  was unstable and did

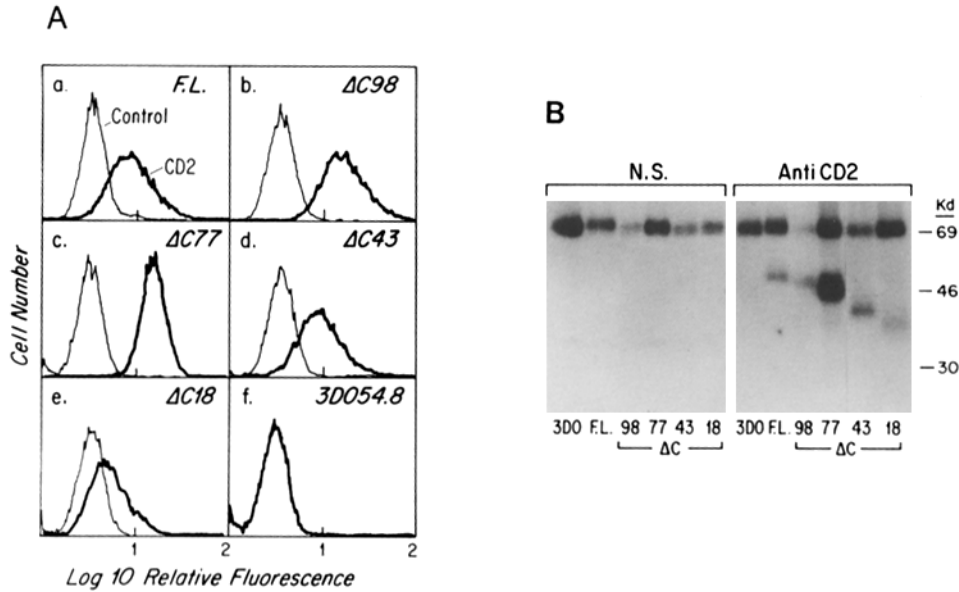


**FIGURE 1.** Production and analysis of the human CD2 cytoplasmic domain. (a) Comparison of predicted transmembrane and cytoplasmic domain sequences of human and mouse CD2. Amino acid residues are designated in single-letter code with the transmembrane regions underlined. Identical residues in human and mouse CD2 sequences are starred. (b) Schematic structure of the transmembrane and cytoplasmic regions of human CD2 and variant molecules. Constructs of full-length, deletion, and substitution mutants of CD2 are diagrammed. The region most conserved between human and mouse CD2 is stippled, and the two repeating PPPGHR segments are marked in black. The H denotes the histidine residues thought to form a putative binding site. The restriction sites that generate the truncated CD2 molecules are marked by arrows with numbers in parentheses corresponding to amino acid residues. (c) Structure of the DOL retroviral expression vector. Selected restriction sites on the vector shown are: B, Bam HI; RI, Eco RI; RV, Eco RV; H3, Hind III. The vector contains two promoters: MLV-LTR to drive the expression of the CD2 cDNA and the SV40 promoter to express the neomycin resistance gene (24).

not allow functional analysis (data not shown). For each clone, similar reactivities to those obtained with anti-T11<sub>1</sub> were found using the anti-T11<sub>2</sub> antibody, while none of the unactivated clones was stained by the anti-T11<sub>3</sub> antibody (data not shown).

To prove that individual clonal recipients of the truncated CD2 cDNAs express appropriately sized CD2 proteins, immunoprecipitation and SDS-PAGE analysis of the corresponding <sup>125</sup>I-labeled surface CD2 molecules was carried out. As expected, no human CD2 was immunoprecipitated from the murine 3DO54.8 cell line. In contrast, a 53-kD band was identified in SDS-PAGE analysis of clone CD2 FL. Note that the 70 kD band in anti-CD2 precipitations is unrelated to CD2 as it is present in the control immunoprecipitates. Furthermore, parallel analysis of CD2 protein expressed by CD2 ΔC98, ΔC77, ΔC43, and ΔC18 revealed that the molecular weights of surface CD2 (51, 47, 43, and 40 kD, respectively) correlated well with the expected truncations (Fig. 2 b). Thus, the truncated CD2 cDNAs in DOL direct protein synthesis of the variant CD2 forms on the surface of the murine 3DO54.8 cells.

Given that perturbation of the external domain of the CD2 molecule with a combi-



**FIGURE 2.** Expression of human CD2 molecules on murine T cells. (a) Flow cytometric analysis of CD2 expression on murine T cells. Indirect immunofluorescence assays were carried out using the anti-T11<sub>1</sub> mAb (3T4-8B5) (thick line) and compared with an irrelevant antibody (1HT4-4E5) (thin line) as background. (b) Immunoprecipitation of CD2 from lysates of iodinated cell lines. Immunoprecipitates were obtained from solubilized murine T cells using a nonspecific (NS) antibody (mouse anti-human CD8) or an anti-CD2 antibody directed against the T11<sub>1</sub> epitope and run under reducing conditions over an SDS 10% polyacrylamide gel (PAGE). The resulting autoradiogram is shown. A contaminant band of 70 kD (as well as material resolving at 200 kD, not shown) was regularly detected in the autoradiograms despite extensive preclearing, as previously reported by others (20).

nation of anti-T11<sub>2</sub> + anti-T11<sub>3</sub> results in a rapid rise in  $[Ca^{2+}]_i$  linked to IL-2 gene induction (1, 9), we examined whether such a mitogenic combination of mAbs was effective in stimulation of CD2 FL as well as CD2  $\Delta C$  cell lines. Fig. 3 a shows an analysis of alteration in  $[Ca^{2+}]_i$  after stimulation with anti-T11<sub>2</sub> + anti-T11<sub>3</sub> in various cell lines as measured with a calcium sensitive dye indo-1 (22) and flow cytometric analysis in real time. A clear rise in  $[Ca^{2+}]_i$  ( $\sim 200$  nM increment) was observed upon stimulation of CD2 FL, CD2  $\Delta C98$  and CD2  $\Delta C77$  cells. The calcium rise occurs within 2 min after adding the stimulating antibodies, most likely corresponding to the time required for expression of the T11<sub>3</sub> epitope after anti-T11<sub>2</sub> stimulation, a phenomena observed previously for human T lymphocytes (1). In contrast, CD2  $\Delta C43$  and CD2  $\Delta C18$  clones were not triggered by anti-T11<sub>2</sub> + anti-T11<sub>3</sub> antibodies. As expected, the nontransfected line 3DO54.8 was also not stimulated. Given that an immediate  $[Ca^{2+}]_i$  rise was observed after addition of the  $Ca^{2+}$  ionophore A23187 (1  $\mu g/ml$ , final concentration), it is clear that cells were loaded with the fluorescent dye. These data establish that a significant rise in  $[Ca^{2+}]_i$  can be induced through human CD2 structures expressed on the membrane of murine T cells even in the absence of the COOH-terminal 40 amino acid residues of the CD2 cytoplasmic segment. The present results with human CD2 are consistent with two

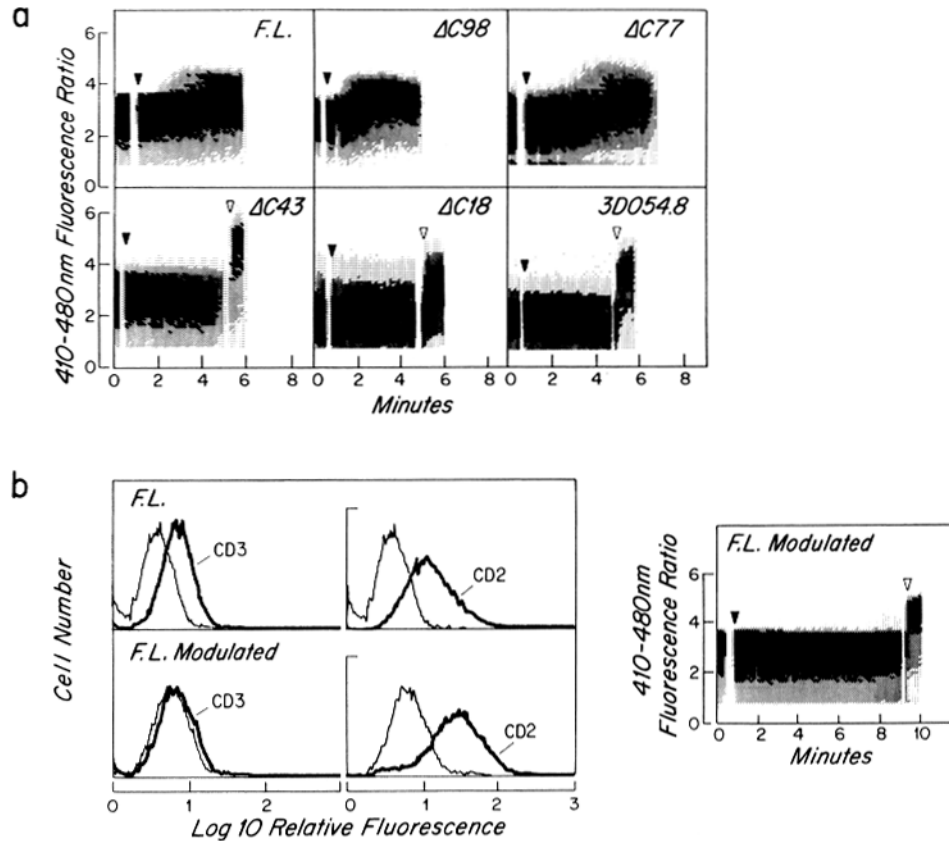


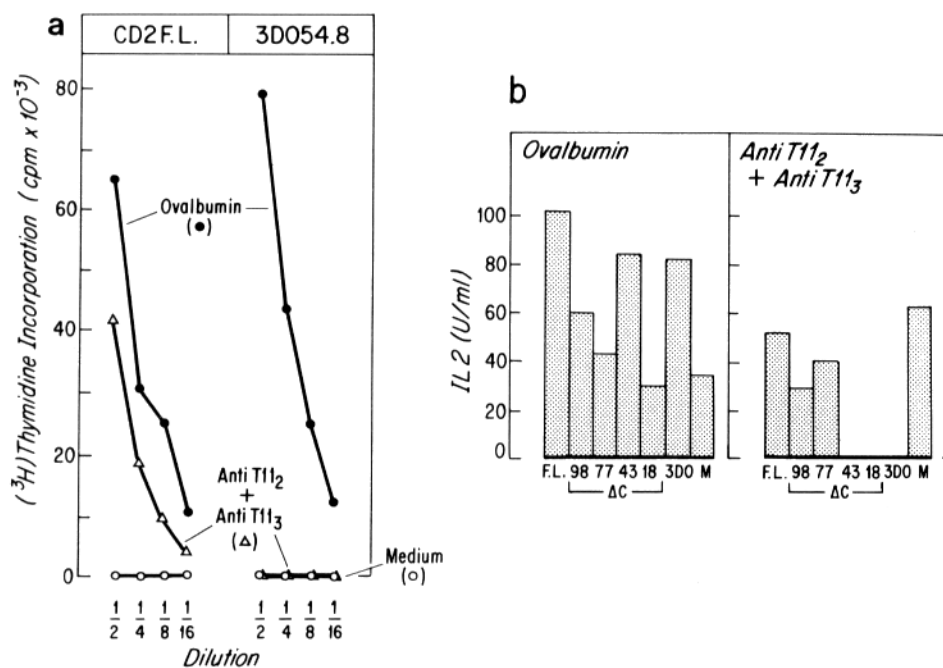
FIGURE 3. Elevation in intracellular free calcium mediated by CD2 stimulation. (a) Measurement of cytosolic  $\text{Ca}^{2+}$  by indo-1 fluorescence. Indo-1 fluorescence changes in real time histograms of T cell lines. Black and open arrows correspond to addition of anti-T11<sub>2</sub> + anti-T11<sub>3</sub> antibodies and calcium ionophore A23187, respectively. (b) Influence of CD3 modulation on induction of  $[\text{Ca}^{2+}]_i$ ; elevation triggered through the human CD2 molecule. Left panel represents indirect immunofluorescence detection of murine CD3 and human CD2 molecule, respectively, on the surface of unmodulated CD2 FL (*top*) and modulated CD2 FL (*bottom*). The latter was incubated with the 145 2C11 monoclonal antibody overnight (28). Right panel represents measurement of  $[\text{Ca}^{2+}]_i$  vs. time in modulated CD2 FL cells after stimulation with anti-T11<sub>2</sub> + anti-T11<sub>3</sub> antibodies (*filled arrow*) or calcium ionophore (*open arrow*).

independent reports. He et al. (25) demonstrated a requirement for the cytoplasmic tail of rat CD2 to trigger a rise in  $[\text{Ca}^{2+}]_i$  after transfection of rat CD2 in the human Jurkat T cell line. Bierer et al. (26) demonstrated a requirement for the cytoplasmic tail of human CD2 expressed in murine T cells for an IL-2 response to liposomes containing both HLA-DR and LFA-3 molecules.

Since human CD2 function in human T lymphocytes requires expression of the CD3-Ti  $\alpha/\beta$  complex (1, 3, 27), we examined whether the function of a human CD2 molecule within a murine cell line was linked to murine CD3-Ti. As shown in Fig. 3 *b*, modulation of the murine CD3 molecule by the hamster anti-mouse CD3 $\epsilon$  antibody (145 2C11) resulted in nearly complete loss of the CD3 molecule from the cell surface of CD2 FL after incubation for 16 h at 37°C, while CD2 expression

was unaltered or slightly increased. Such anti-CD3 modulated CD2 FL cells were no longer stimulated by anti-T11<sub>2</sub> + anti-T11<sub>3</sub> antibodies to increase [Ca<sup>2+</sup>]<sub>i</sub>. By contrast, the incubation of CD2 FL cells under similar conditions with anti-human CD3 antibody (Leu4) did not have any effect (Materials and Methods). This result suggests that regulation of the CD2 pathway by CD3-Ti is intact in the cellular model herein, further supporting the validity of this functional approach in the study of the CD2 intracellular domain. We cannot, of course, exclude the possibility that CD3 modulation leads to a generalized disruption of subsequent T cell activation.

We next examined if nuclear activation events including IL-2 induction and subsequent IL-2 secretion could be triggered through the human CD2 molecule in murine CD2 FL cells. To this end, clones were stimulated and IL-2 secretion into supernatants assayed using the IL-2-dependent CTLL-20 cells (29). As shown in Fig. 4 *a*, supernatants from CD2 FL or 3DO54.8 cells stimulated with ovalbumin in the presence of the H-2(I-A<sup>d</sup>) expressing A20-11 B lymphoma are able to induce proliferation of the IL-2-dependent CTLL-20 cells in a comparable way. By contrast, the



**FIGURE 4.** Stimulation of IL-2 production through the human CD2 molecule and its variants forms on murine T cells. (a) Effect of culture supernatants from CD2 FL or 3DO54.8 on proliferation of CTLL-20 cells. Serial twofold dilutions of culture supernatants from stimulated or unactivated murine cells were tested for their capacity to support the growth of the IL-2-dependent murine CTLL-20 cell line (29), as evaluated by [<sup>3</sup>H]thymidine incorporation. (b) IL-2 production upon antigen (ovalbumin) or anti-T11<sub>2</sub> + anti-T11<sub>3</sub> stimulation. Under these experimental conditions, the limit of detection for IL-2 was <4 U/ml. M represents the murine T cell line CD2 M271-2. Analysis of IL-2 production by two independent clones of the CD2 M278-9 provided evidence that such cells could produce significant amounts of IL-2 upon CD2 triggering (~50% of the amount secreted upon antigen stimulation). Experiments were performed as above, but precise quantification of IL-2 production was obtained by running culture supernatants in parallel to a titration curve of rIL-2 (Biogen Laboratories, Cambridge, MA).

combination of anti-T11<sub>2</sub> + anti-T11<sub>3</sub> antibodies is effective in inducing IL-2 production by CD2 FL cells but not the parental line 3DO54.8. Fig. 4 *b* shows that all of the cell lines tested including CD2 FL, 3DO54.8, and the CD2 ΔC series produce a high amount of IL-2 (ranging from 30 to 100 U/ml, corresponding to a clonal variation repeatedly observed) when stimulated with ovalbumin in the I-A<sup>d</sup> context. This result demonstrates the integrity of the IL-2 synthetic pathway in each clone. Perhaps more importantly, after stimulation with anti-CD2 antibodies, the clones CD2 FL, CD2 ΔC98, and CD2 ΔC77 produce comparable levels of IL-2, while transfectants CD2 ΔC43 and CD2 ΔC18, like the untransfected 3DO54.8, are not triggered through CD2. Analysis of five other independent clones expressing the ΔC43 CD2 molecule clearly established that these cells are not triggered through human CD2 either to secrete detectable levels of IL-2 or to elevate [Ca<sup>2+</sup>]<sub>i</sub> (data not shown). Taken together, these data show that a full-length human CD2 molecule, as well as a CD2 molecule lacking 19 or 40 COOH-terminal amino acids from the cytoplasmic domain, is able to activate T lymphocytes after appropriate perturbation of the CD2 extracellular segment. Interestingly, the CD2 ΔC77 clones express human CD2 molecules lacking residues 289 to 316. The latter corresponds to the segment most conserved among human and murine molecules, with 24 out of 27 residues being identical (Fig. 1, *a* and *b*). Presumably, these conserved residues function in another facet of CD2 biology unrelated to IL-2 induction and/or secretion. In contrast, the ΔC43 truncated molecules as well as shorter truncations are nonfunctional with respect to stimulating a rise in [Ca<sup>2+</sup>]<sub>i</sub> and IL-2 production.

These data provide unequivocal evidence that the CD2 cytoplasmic domain is involved in signal transduction and that one essential sequence of the cytoplasmic domain necessary for CD2-mediated activation is located between amino acids 253 to 287. This region contains four histidines at amino acid positions 264, 271, 278, and 282 and includes two tandemly repeated segments (PPPGHR, amino acids 260–265 and 274–279) (see Fig. 1, *a* and *b*). These histidine residues could represent a binding site for an ion, cyclic nucleotide, or other small regulatory molecule.

To evaluate the role of the histidine residues, substitution mutants of CD2 were produced by site-directed mutagenesis as described in Materials and Methods. Two different categories of mutants with a stable CD2 surface expression were obtained: CD2 M271-2 and CD2 M278-9 in which the positively charged histidine and arginine residues at position 271 and 272 or 278 and 279 were replaced by negatively charged aspartic acid and glutamic acid, respectively (Fig. 1 *b*). Functional characterization of these two mutants shows that IL-2 production can be induced by anti-T11<sub>2</sub> and anti-T11<sub>3</sub> antibodies to a level comparable to that of the CD2 FL clones (Fig. 4 *b* and data not shown). Thus, a putative “cage,” requiring four histidine residues, is not necessary for this CD2 activation. Furthermore, since the mutations at position 278–279 alter the structure of the second repeat without affecting T cell activation, the more COOH-terminal repeat is not required. Therefore, it may be speculated that perhaps only one PPPGHR sequence is necessary for induction of IL-2 activity. This notion can be tested by further site-directed mutagenesis studies. Alternatively, the repeats and/or other histidine residues could function to regulate CD2 interaction with cytoskeletal, plasma membrane, cytosolic, nuclear, or other cellular components not examined by the present assays. It also remains to be determined if the CD2 cytoplasmic region influences the CD2 extracellular segment and



hence, interaction with LFA-3. The present model system will be useful at addressing these latter possibilities.

### Summary

To evaluate those residues in the 117 amino acids of the CD2 cytoplasmic domain required for transduction of T lymphocyte activation signals, a full-length human CD2 cDNA and a series of deletion and substitution mutants were inserted into the ovalbumin-specific, I-A<sup>d</sup>-restricted murine T cell hybridoma 3DO54.8 using a retroviral system. The resulting cells express surface CD2 protein and unlike the parental murine line, are reactive with murine anti-human CD2 antibodies. Anti-T11<sub>2</sub> plus anti-T11<sub>3</sub> antibody stimulation of cells expressing a full-length CD2 cDNA results in a characteristic rise in cytosolic-free calcium ( $[Ca^{2+}]_i$ ), and subsequent IL-2 secretion that accompany CD2 stimulation in human T lymphocytes. Transfectants expressing CD2  $\Delta$ C98 and CD2  $\Delta$ C77, partially deleted CD2 molecules containing the entire extracellular and transmembrane CD2 segments but only 98 and 77 amino acids of the cytoplasmic domain, respectively, are also activated by anti-CD2 mAbs. In contrast, clones expressing more severely truncated CD2 structures, CD2  $\Delta$ C43 and CD2  $\Delta$ C18, are not stimulated. These data show that the cytoplasmic domain plays an essential role in transduction of activation signals via CD2, and that the segment between amino acid residues 253 and 278 is necessary for activation. This region contains two tandem repeats of the sequence PPPGHR, thought to form part of a putative cationic site. Disruption of the latter by site-directed mutagenesis does not affect IL-2 gene induction, suggesting that only one of the repeats is required for activating this function of the CD2 molecule.

*Received for publication 30 November 1988 and in revised form 16 February 1989.*

### References

1. Meuer, S. C., R. E. Hussey, M. Fabbi, D. Fox, O. Acuto, K. A. Fitzgerald, J. C. Hodgdon, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz. 1984. An alternative pathway of T cell activation: a functional role for the 50KD T11 sheep erythrocyte receptor protein. *Cell*. 36:897.
2. Siliciano, R. F., J. C. Pratt, R. E. Schmidt, J. Ritz, and E. L. Reinherz. 1985. Activation of cytolytic T lymphocytes and natural killer cell function through the T11 sheep erythrocyte binding protein. *Nature (Lond.)*. 317:428.
3. Brottier, P., L. Boumsell, C. Gelin, and A. Bernard. 1985. T cell activation via CD2 (T, gp50) molecules: accessory cells are required to trigger T cell activation via CD2-D66 plus CD2-9.6/T11 (1) epitopes. *J. Immunol.* 135:1624.
4. Selvaraj, P., M. L. Plunkett, M. Dustin, M. E. Sanders, S. Shaw, and T. A. Springer. 1987. The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3. *Nature (Lond.)*. 326:400.
5. Dustin, M. L., M. E. Sanders, S. Shaw, and T. A. Springer. 1987. Purified lymphocyte function-associated antigen 3 binds to CD2 and mediates T lymphocyte adhesion. *J. Exp. Med.* 165:677.
6. Yang, S. Y., S. Chouaib, and B. DuPont. 1986. A common pathway for T lymphocyte activation involving both the CD3-Ti complex and the CD2 sheep erythrocyte receptor determinants. *J. Immunol.* 137:1097.
7. Bierer, B. E., J. Barbosa, S. Herrmann, and S. J. Burakoff. 1988. The interaction of

- CD2 with its ligand, LFA-3, in human T cell proliferation. *J. Immunol.* 140:3358.
8. Alcover, A., M. J. Weiss, J. F. Daley, and E. L. Reinherz. 1986. The T11 surface glycoprotein is functionally linked to a calcium channel in precursor and mature T lineage cells. *Proc. Natl. Acad. Sci. USA.* 83:2614.
  9. Pantaleo, G., D. Olive, A. Poggi, W. J. Kozumbo, L. Moretta, and A. Moretta. 1987. Transmembrane signalling via the T11-dependent pathway of human T cell activation. Evidence for the involvement of 1,2-diacylglycerol and inositol phosphates. *Eur. J. Immunol.* 17:55.
  10. Hunig, T., G. Tiefenthaler, K. H. Meyer Zum Buschenfelde, and S. C. Meuer. 1987. Alternative pathway activation of T cells by binding of CD2 to its cell surface ligand. *Nature (Lond.)* 326:298.
  11. Sayre, P. H., H. C. Chang, R. E. Hussey, N. R. Brown, N. E. Richardson, G. Spagnoli, L. K. Clayton, and E. L. Reinherz. 1987. Molecular cloning and expression of T11 cDNAs reveal a novel receptor-like structure on human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 84:2941.
  12. Clayton, L. K., P. H. Sayre, J. Novotny, and E. L. Reinherz. 1987. Murine and human T11 (CD2) cDNA sequences suggest a common signal transduction mechanism. *Eur. J. Immunol.* 17:1367.
  13. Sewell, W. A., M. H. Brown, J. Dunne, M. J. Owen, and M. J. Crumpton. 1986. Molecular cloning of the T lymphocyte surface CD2 (T11) antigen. *Proc. Natl. Acad. Sci. USA.* 83:8718.
  14. Seed, B., and A. Aruffo. 1987. Molecular cloning of the CD2 antigen, the T cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA.* 84:3365.
  15. Diamond, D. J., L. K. Clayton, P. H. Sayre, and E. L. Reinherz. 1988. Exon-intron organization and sequence comparison of human and murine T11 (CD2) genes. *Proc. Natl. Acad. Sci. USA.* 85:1615.
  16. Lang, G., D. Wotton, M. J. Owen, W. A. Sewell, M. H. Brown, D. Y. Mason, M. J. Crumpton, and D. Kioussis. 1988. The structure of the human CD2 gene and its expression in transgenic mice. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1675.
  17. Sewell, W. A., M. H. Brown, M. J. Owen, P. J. Fink, C. A. Kozak, and M. J. Crumpton. 1987. The murine homologue of the T lymphocyte CD2 antigen: molecular cloning, chromosome assignment and cell surface expression. *Eur. J. Immunol.* 17:105.
  18. Williams, A. F., A. N. Barclay, S. J. Clark, D. J. Paterson, and A. C. Willis. 1987. Similarities in sequence and cellular expression between rat CD2 and CD4 antigens. *J. Exp. Med.* 165:368.
  19. Taylor, J. W., J. Ott, and F. Eckstein. 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothionate-modified DNA. *Nucleic Acids Res.* 13:8764.
  20. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J. Exp. Med.* 157:1149.
  21. Cepko, C. L., B. E. Roberts, and R. C. Mulligan. 1984. Construction and applications of highly transmissible murine retrovirus shuttle vector. *Cell.* 37:1053.
  22. Grynkiewicz, G., M. Poenic, and R. Y. Tsien. 1985. A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440.
  23. Pantaleo, G., D. Olive, A. Poggi, T. Pozzari, L. Moretta, and A. Moretta. 1987. Antibody-induced modulation of the CD3/T cell receptor complex causes T cell refractoriness by inhibiting the early metabolic steps involved in T cell activation. *J. Exp. Med.* 166:619.
  24. Korman, A. J., J. D. Frantz, J. L. Strominger, and R. C. Mulligan. 1987. Expression of human class II major histocompatibility complex antigen using retrovirus vectors. *Proc. Natl. Acad. Sci. USA.* 84:2150.

25. He, Q., A. D. Beyers, A. N. Barclay, and A. F. Williams. 1988. A role in transmembrane signalling for the cytoplasmic domain of the CD2 T lymphocyte surface antigen. *Cell*. 54:979.
26. Bierer, B. A., A. Peterson, J. C. Gorga, S. H. Herrmann, and S. J. Burakoff. 1988. Synergistic T cell activation via the physiological ligands for CD2 and the T cell receptor. *J. Exp. Med.* 168:1145.
27. Alcover, A., C. Alberini, O. Acuto, L. K. Clayton, C. Transy, G. Spagnoli, P. Moingeon, P. Lopez, and E. L. Reinherz. 1988. Interdependence of CD3-Ti and CD2 activation pathways in human T lymphocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1973.
28. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84:1374.
29. Gillis, S., M. Ferm, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2077.