Dependence of T Cell Antigen Recognition on the Dimensions of an Accessory Receptor–Ligand Complex

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Summary

The T cell antigen receptor (TCR) and its ligand peptide–major histocompatibility complex (MHC) are small (~7 nm) compared with other abundant cell surface molecules such as integrins, CD43, and CD45 (23–50 nm). We have proposed that molecules at the T cell/antigen-presenting cell (APC) interface segregate according to size, with small "accessory" molecules (e.g., CD2, CD4, CD8, CD28, and CD154) contributing to the formation of a close-contact zone, within which the TCR engages peptide–MHC, and from which large molecules are excluded (Davis, S.J., and P.A. van der Merwe. 1996. *Immunol. Today*. 17:177–187). One prediction of this model is that increasing the size of these small accessory molecules will disrupt their function. Here, we test this prediction by varying the dimensions of the CD2 ligand, CD48, and examining how this affects T cell antigen recognition. Although the interaction of CD2 on T cells with wild-type or shortened forms of CD48 on APCs enhances T cell antigen recognition, the interaction of CD2 with elongated forms of CD48 is strongly inhibitory. Further experiments indicated that elongation of the CD2/CD48 complex inhibited TCR engagement of peptide–MHC, presumably by preventing the formation of sufficiently intimate contacts at the T cell/APC interface. These findings demonstrate the importance of small size in CD2/CD48 function, and support the hypothesis that T cell antigen recognition requires segregation of cell surface molecules according to size.

Key words: T cell receptor • CD2 • CD48 • T cell activation • plasma membrane

T cell antigen recognition requires an interaction between the TCR and a complex of peptide antigen with MHC molecules (peptide–MHC) on the surface of APCs or target cells. This interaction takes place at the contact interface between a T cell and an APC or target cell. It has been pointed out (1, 2) that both the TCR (3, 4) and peptide–MHC (5, 6) are unusually small (~7 nm) compared with highly abundant cell surface molecules such as integrins (~25 nm), CD43 (~43 nm), or CD45 (23–50 nm) (7). This raises the question as to how the plasma membranes of the T cell and APC or target cell form contacts that are sufficiently intimate (~15 nm between membranes) for the TCR to engage peptide–MHC (8, 9).

Several T cell "accessory" molecules implicated in antigen recognition (e.g., CD2, CD4, CD8, CD28, and CD154) are comparable in size to the TCR (7, 10). CD2 is arguably the best characterized of these molecules (11–13). It has two Ig-like domains in its extracellular portion (14, 15) and a highly conserved proline-rich cytoplasmic domain that has been shown to bind SH3 domains of cytoplasmic proteins (16–18). The extracellular portion of CD2 mediates cell–cell recognition by binding to CD58 (LFA-3) in humans (19, 20) and CD48 in rodents (21, 22). CD2, CD48, and CD58 are members of the CD2 family of Ig superfamily molecules, which includes 2B4 and CD150 (SLAM) (13). CD2 family members are structurally related and expressed by linked genes (7, 23), suggesting that they have evolved from a common ancestral molecule capable of homotypic adhesion (24).

Although numerous studies have shown that the interaction between CD2 and its ligands can enhance T cell antigen recognition (12, 25, 26), the observation that CD2-deficient mice have relatively mild abnormalities in T cell development and T cell function (27–29) indicates that CD2 is not indispensable. It has been suggested that T cells are able to adapt during development to function in the ab-

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sence of CD2 (by selection of a high-affinity TCR repertoire) or that other molecules can fulfill the same function (27). Although there is convincing evidence that CD2 enhances T cell antigen recognition by mediating adhesion of T cells to APCs or target cells (11, 12, 25), it may also transduce signals through its cytoplasmic domain (12, 25, 26, 30). Structural (14) and mutagenesis (8) studies indicate that CD2 binds to CD48 in a head-to-head orientation such that the CD2/CD48 complex is likely to be of similar length (~14 nm) to the TCR/peptide–MHC complex (3, 31). This has led to the proposal that the CD2/ligand interaction may facilitate T cell antigen recognition by participating in the formation of (a) close-contact zone(s) within the contact interface between T cells and APCs or target cells in which the intermembrane separation distance is optimal for TCR engagement of peptide–MHC (8, 9, 13).

Here, we test this hypothesis by varying the length of the CD2 ligand, CD48, and examining the effect this has on T cell antigen recognition. We show that whereas wild-type or shortened CD48 enhanced T cell antigen recognition, long forms of CD48 were profoundly inhibitory, preventing TCR engagement of peptide–MHC. These results demonstrate the important role size plays in the accessory function of the CD2/CD48 interaction, and support the notion that close membrane approximation (<21 nm) is a prerequisite for T cell antigen recognition.

Materials and Methods

Cells and Antibodies. The 2B4 T cell hybridoma and its derivatives 2B4.CD2 (2B4.mCD2-1.6; transfected with full-length mouse CD2) and 2B4.CD2trunc (2B4.mCD2.M3-2.1; transfected with cytoplasmic tail–deleted mouse CD2) have been described previously (26, 32). They were cultured in RPMI-10 medium (RPMI 1640 [GIBCO BRL], 10% FCS [Sigma Chemical Co.], 100 U/ml penicillin, 100 μg/ml streptomycin [GIBCO BRL], 4 mM glutamine [GIBCO BRL], and 5×10^{-5} M 2-ME [Sigma Chemical Co.]). G418 (0.4 mg/ml; GIBCO BRL) was added to medium used for 2B4.CD2 and 2B4.CD2trunc cells in order to maintain expression of CD2. The Chinese hamster ovary (CHO)[1] cell line N1.A4 expressing mouse I-E^k has been described elsewhere (33, 34). It was cultured in RPMI-10 medium containing 0.4 mg/ml G418 to maintain I-E^k expression.

The following mAbs were used: RM2-1, rat anti–mouse CD2, IgG2a (35); OX11, rat anti–rat κ chain, IgG2a (36, 37); OX78, rat anti–mouse CD48, IgG2a (21); IE-D6, mouse anti–mouse I-E^k, IgG2a (Serotec); OX12, mouse anti–rat κ chain, IgG2a (36); 145-2C11, hamster anti–mouse-CD3 (38); A2B4-2, mouse anti-2B4 TCR-α, IgG2a (39); and 30-H12, rat anti–mouse Thy1.2, IgG2b (PharMingen).

Expression of CD48 Constructs in CHO Cells. Four different mouse CD48 constructs were made: wild-type CD48 with a silent BssHII site NH_2-terminal to the glycosyl phosphatidylinositol (GPI) signal sequence for the insertion of additional domains; elongated molecules with two-domain human CD2 (CD48-CD2) or three-domain mouse CD22 (CD48-CD22) inserts; and a shortened CD48 construct (CD48d1) in which the membrane-proximal domain (d2) had been deleted (see Fig. 1 A). The wild-type CD48 construct was made by PCR in two steps using as template the vector pCD.MBCM-1, which contained the sequence for full-length mouse CD48 (23). In the first PCR, the 5′ primer 1130 (tagtagtctagaccccatccgctcaagcaggccaccATGTGCTTCATAAAACAGGGATGGTG) encoded an XbaI site (restriction sites are underlined in all primers), the rat CD4 5′-untranslated region (in lower case), and the first 26 nucleotides of the CD48 sequence beginning with the start codon (upper case; compare with Wong et al. [23]). The 3′ primer GTTGTGACCACTAGCCAAGTTGCAGTCCAACATACTCCAGAAGAgCgcGCTAGATCACAAGGTAG encoded for the 3′ end of the second domain of CD48, a silent restriction site (BssHII), and part of the GPI anchor signal sequence. The resulting product was elongated in a second PCR using the same 5′ primer (1130) with the 3′ primer ctactagGaTCcTCAGGTTAACAaGATCCTGTGAATGATGAGTGTTGTGACCACTAGCCAAGTTGC. This primer overlaps the 3′ primer used in the first PCR and encodes for the remainder of the CD48 sequence followed by a BamHI site. In addition, a silent mutation (lower case "a") was introduced to eliminate an internal BamHI site. The PCR product was digested with XbaI and BamHI and ligated into vector pEF-BOS-XB (40).

To obtain a DNA fragment encoding domains 1 and 2 of human CD2 which could be inserted into the silent BssHII site in the wild-type CD48 construct, a plasmid encoding the extracellular portion of human CD2 (41) was used as template for a PCR with the 5′ primer tagtaggcgcgcGAGATTACGAATGCCTTGGAAACC, which encodes a BssHII site and the CD2 signal sequence (42), and the 3′ primer ctactagcgcgCTTTCTCTGGACAGCTGACAGG, which encodes the final 21 nucleotides of domain 2 (42) flanked by a BssHII site.

The mouse CD22 domain 1–3 insert was generated by PCR using as template a plasmid encoding a CD22(d1–3) Fc chimera with the mutation, R130A (43). The primers introduced flanking BssHII sites: 5′ primer, tagtaggcgcgcGATTGGACCGTTGACCATC; 3′ primer, ctactagcgcgcGTGCACCGTGAGTTCCAC. The CD2 and CD22 fragments were cloned into the BssHII site in the CD48 wild-type pEFBOS-XB plasmid. In the final construct, the junctions at the CD2 insert were TGTGATCTAGCGCGCgagattacg and ccagagaaaGCGCGCTCTTCT (CD48 in upper case, CD2 in lower case, and BssHII site underlined). Similarly, the junctions at the CD22 insert were GATCTAGCGCGCgattggacc and gtgcacGCGCGCTCTTCT.

The CD48d1 construct was obtained by PCR with the CD48 wild-type construct as template, using 1130 as 5′ primer and ctactagcgcgCAGGATCAAATACTTCCAG as 3′ primer. The latter encoded for the last nucleotides of the membrane-distal domain of CD48 and added a BssHII site. The PCR product was digested with XbaI and BssHII and inserted into the CD48 wild-type pEFBOS-XB plasmid in place of the fragment encoding CD48 d1 and 2. The boundary between domain 1 and the mCD48 GPI anchoring signal in the resulting construct was TTTGATCCTGCGCGCTCTTCT. All constructs were sequenced using an Applied Biosystems DNA sequencing system (model 373A).

For expression, the CD48 constructs were subcloned into the pEE14 expression vector using its XbaI and BclI sites (44, 45). N1.A4 CHO cells were transfected and selected using the glutamine synthetase selection system (44, 45). Stable lines were selected at 15–40 μM l-methyl sulfoximine (Sigma Chemical Co.) and recloned. Clones which gave comparable staining with CD48 mAb in cytometry analyses were chosen for functional as-

[1]*Abbreviations used in this paper:* CHO, Chinese hamster ovary; GPI, glycosyl phosphatidylinositol; ICAM, intercellular adhesion molecule; MCC, moth cytochrome c.

says. They were maintained in culture in CB2-10 medium (CB-2 [GIBCO BRL], 10% dialyzed FCS [First Link], 100 U/ml penicillin and 100 μg/ml streptomycin [GIBCO BRL], 0.4 mg/ml G418 [Sigma Chemical Co.], and 15–40 μM l-methyl sulfoximine). Revertants of the various clones were obtained by culturing cells for 8–12 wk in the absence of l-methyl sulfoximine and sorting for CD48$^-$ cells on a FACSort™ cell sorter (Becton Dickinson).

Western Blotting. Triton X-100 lysates of 10^5 CHO cells per lane were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose (Hybond-C-super; Amersham Pharmacia Biotech). Membranes were probed with 1:7 dilutions of mAb OX78 and OX11 spent tissue culture supernatant, followed by a 1:2,000 dilution of peroxidase-conjugated rabbit anti–rat Ig serum (DAKO) and developed using the ECL+Plus system (Amersham Pharmacia Biotech). Purified soluble mouse CD48 was used as a positive control (46).

Flow Cytometry. Standard procedures were used to stain cells with purified mAbs (15 μg/ml) or hybridoma culture supernatants. Binding of CD2 chimeric protein to CHO cells was assessed using a previously described method developed for detection of low-affinity interactions (47). In brief, 12.5 μl avidin-coated fluorescent beads (YFP-0552-5; Spherotech) per sample were rotated with 1.25 μg biotinylated anti–human Fcγ mAb SB2H2 (47) and 40 μl of PBS/0.2% BSA for 45 min at 4°C, washed in PBS/0.02% BSA, and rotated with 3 μg/ml of human CD2 Fc (21) or human CTLA-4 Fc (48) for 2 h at 4°C in 1 ml of RPMI-10 medium. Beads were washed and resuspended in 12.5 μl PBS/BSA per sample, sonicated for 1 min, and added to 40 μl PBS/BSA containing 10^5 CHO cells in a well of a flat-bottomed microtiter plate. Plates were centrifuged for 20 min at 1,000 rpm at 4°C and incubated at 0°C for 40 min before the cells were resuspended in 0.5 ml PBS/BSA and analyzed in a FACScan® flow cytometer (Becton Dickinson).

Binding of CD48 to T cells was measured in a similar assay but using a chimeric protein consisting of mouse CD48 fused to rat CD4 domains 3 plus 4, followed by a short peptide tag which can be enzymatically biotinylated (40). To create this construct, an XhoI site was inserted 5′ of the His tag in a mouse sCD48his encoding plasmid (46) to enable the sCD48 portion to be excised with XbaI and XhoI and cloned into the XbaI/SalI sites of a vector encoding rat CD4d3+4 and a consensus peptide sequence recognized by the *Escherichia coli* biotin holoenzyme synthetase, BirA (40). The CD48-CD4 boundary in the resulting chimera was CTAGCCcgctcgacATCCATC. A rat CD5-CD4d3+4–biotin construct was made in an analogous way. Both constructs were expressed and biotinylated as described (40). The protein-coated beads were prepared by rotating 12.5 μl of avidin-coated fluorescent beads with 1 μg biotinylated protein in 350 μl PBS/BSA for 1 h at 4°C, followed by washing, sonication, and cell labeling as described above.

T Cell Activation Assays. In each well of a V-bottomed microtiter plate (Bibby Sterilin Ltd.), 5×10^4 2B4 cells were mixed with 5×10^4 irradiated (3,000 rad) CHO cell APCs and different concentrations of HPLC-purified moth cytochrome c (MCC) peptide 88–103 (ANERADLIAYLKQATK; made by Dr. N.P. Groome, Oxford Brookes University, Oxford, UK) and cultured in RPMI-10 medium for 18 h. In some experiments, CHO cells were preincubated with 10 μg/ml of mouse CD48 mAb OX78. This concentration of mAb was maintained during the 2B4/APC/peptide coculture. Subsequently, supernatants were harvested, and 2×10^4 per well of IL-2–responsive CTLL-2 cells (American Type Culture Collection) were cultured with serial dilutions of the supernatants in RPMI-10 medium for 18 h. Purified recombinant mouse IL-2 was used as the standard (rec. mIL-2; Boehringer Mannheim). Wells were pulsed with 0.5 μCi/well of [^{3}H]thymidine (Amersham Pharmacia Biotech) and harvested after an additional 7 h. Linear regression (Cricket Graph; Computer Associates International) was used to generate a linear fit, which was used to calculate the unknown IL-2 concentrations. Probably as a consequence of clonal variation, the different 2B4 cells exhibited somewhat different levels of antigen-induced IL-2 production even in the absence of CD2 ligation. This effect had been noted previously in a study with T cells transfected with different forms of human CD2 (49). To allow comparison of the effects of CD48 expression on APCs between different 2B4 clones, the results in some experiments were normalized, letting 100% equal maximal peptide-stimulated IL-2 secretion in the presence of CD48$^-$ CHO cells.

For phorbol ester/calcium ionophore stimulation, peptide was replaced with 50 ng/ml PMA (Sigma Chemical Co.) and 500 ng/ml ionomycin (Sigma Chemical Co.). For CD3 mAb stimulation, flat-bottomed microtiter plates (Falcon) were incubated with serum-free tissue culture supernatant containing CD3 mAb 145-2C11 for 2 h and washed with RPMI-10 before cells were added and cultured as described above.

TCR Downmodulation Assay. 10^5 CHO cell APCs were incubated with MCC peptide 88–103 for 2 h in round-bottomed

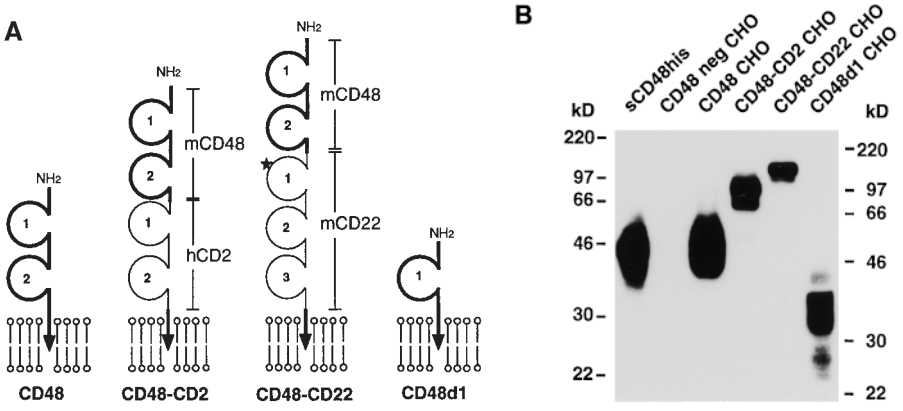

Figure 1. Expression of elongated and shortened forms of CD48 in I-E^{k+} CHO cells. (A) Schematic representation of the various forms of CD48 used in this study. Segments derived from mouse CD48 and segments inserted from human CD2 or mouse CD22 are depicted as heavy and light lines, respectively. The asterisk represents the CD22 mutation, R130A. (B) Western blot of the CD48 constructs expressed on I-E^{k+} CHO cells. Triton X-100 lysates of 10^5 I-E^{k+} CHO cells expressing no CD48 (CD48 neg) or the indicated form of CD48 were run under reducing conditions and blotted with the mouse CD48 mAb, OX78. Soluble recombinant mouse CD48 with an oligohistidine tag (sCD48his, 50 ng) was included for comparison. Parallel blots of the same samples with an isotype-matched control mAb (OX11) gave no staining (not shown).

microtiter plates before the addition of 5×10^4 2B4.CD2 cells. After coculture for 3 h at 37°C, the cells were resuspended in PBS containing 0.5 mM EDTA and stained with anti-Thy1.2 and anti-2B4 TCR mAbs, followed by PE-conjugated goat anti–rat IgG (Serotec) and FITC-conjugated goat anti–mouse IgG2a (Southern Biotechnology Associates). TCR expression on the Thy1.2$^+$ cells (i.e., excluding the APCs) was measured using a FACScan® flow cytometer.

Results and Discussion

Expression of Elongated and Shortened Forms of CD48 on APCs. We examined the effect of varying the dimensions of the CD2/ligand complex using an in vitro T cell antigen recognition assay. The responder cells were the 2B4 murine MHC class II–restricted T cell hybridoma (26, 32, 50). The 2B4 TCR recognizes a peptide fragment (88–103) of MCC (MCC 88–103) bound to I-E^k (51). As APCs, we used CHO cells expressing I-E^k (33). These I-E^{k+} CHO APCs were transfected with wild-type, elongated, or shortened forms of the mouse CD2 ligand, CD48 (Fig. 1 A). The elongated forms of CD48 had either two (CD48-CD2) or three (CD48-CD22) Ig domains inserted as spacers between the membrane-proximal Ig domain of CD48 and its GPI signal sequence (Fig. 1 A). The two-domain insert consisted of the ectodomain of human CD2, whereas the three-domain insert comprised the three membrane-distal Ig domains of mouse CD22. Both these inserts are functionally inert: human CD2 does not bind to mouse CD48 (46, 52), and the CD22 insert has a point mutation (R130A) that abolishes binding to its sialoglycoconjugate ligands (43). The short form of CD48 (CD48d1) was produced by deleting the membrane-proximal Ig domain (Fig. 1 A).

A Western blot of transfected I-E^{k+} CHO cells using an anti-CD48 mAb showed that the various CD48 constructs migrated on SDS-PAGE at positions consistent with their predicted molecular mass (Fig. 1 B). Transfected I-E^{k+} CHO clones that expressed equivalent levels of CD48 and H2-E^k were selected for further analysis (Fig. 2 A). The exception was CD48d1 I-E^{k+} CHO cells, which expressed CD48 at 50% of the level of CD48 I-E^{k+} CHO cells (Fig. 2 B). Beads coated with mouse CD2 Fc (21) bound at least as well to I-E^{k+} CHO cells expressing elongated forms of CD48 as to cells expressing wild-type CD48, indicating that the CD48 remained functional (Fig. 2 A). In contrast, binding of these beads to CD48d1 I-E^{k+} CHO cells was reduced by ~90% (Fig. 2 B).

The Interaction between CD2 and Wild-type CD48 Enhances T Cell Antigen Recognition. Although the interaction of human CD2 with its ligand, CD58, has been shown to enhance T cell antigen recognition, such an effect has not been formally demonstrated for the equivalent murine interaction between CD2 and CD48. This is an important point because CD2-deficient mice have surprisingly mild alterations in T cell function (27, 29), and the mouse and rat CD2/CD48 interactions have an ~6-fold lower solution affinity (46, 53) and ~40-fold lower membrane affinity (54) than the human CD2/CD58 interaction (41). Therefore, we

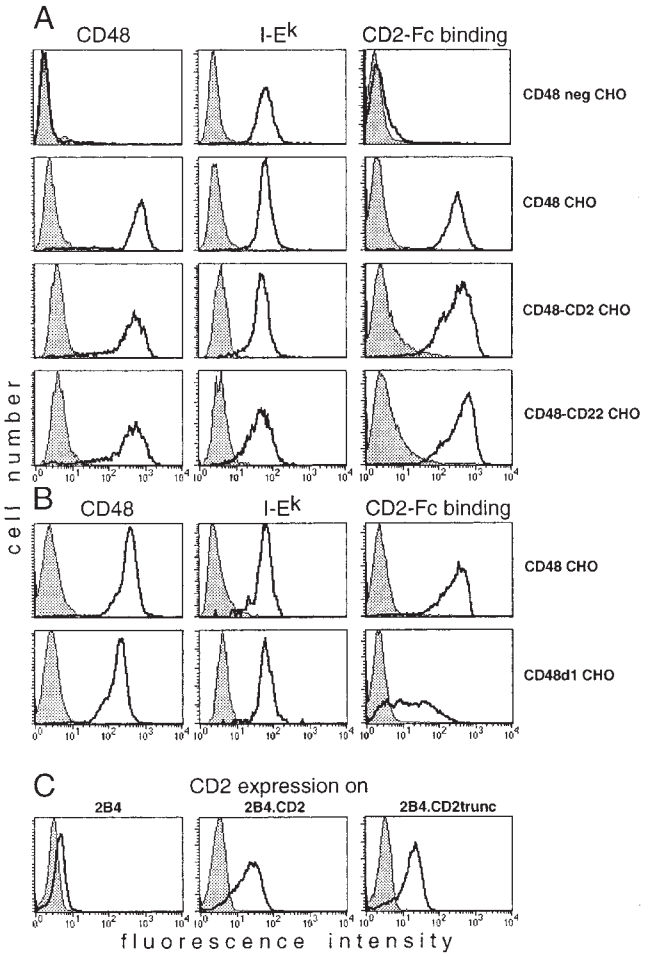

Figure 2. Flow cytometry of APCs and T cells. (A) Comparison of I-E^{k+} CHO cell clones expressing wild-type and elongated forms of CD48. In the left and center columns, cells were labeled with the OX78 (CD48) and IE-D6 (anti–I-E^k) mAbs (thick lines), respectively, or isotype-matched mAbs (left, OX11; middle, OX12) as controls (shaded). In this analysis, OX78 binding was 15–20% lower on CD48-CD2 and CD48-CD22 cells compared with CD48 I-E^{k+} CHO cells. However, no significant difference was measured in three other analyses (not shown). In the right column, cells were labeled with mCD2-Fc–coated (bold line) or hCTLA4-Fc–coated (shaded) fluorescein beads. (B) Comparison of I-E^{k+} CHO cells expressing wild-type and shortened CD48, as in A. (C) Comparison of CD2 expression on the different 2B4 cell lines used. Labeling by the CD2 (RM2-1) and the isotype-matched control (OX11) mAbs is represented by bold lines and shaded histograms, respectively. All 2B4 cells expressed virtually identical levels of surface TCR (not shown).

examined the effect that CD48 expression on I-E^{k+} CHO cells had on antigen recognition by 2B4 T cells. Because the original 2B4 hybridoma expressed only very low levels of CD2 (Fig. 2 C), we used cells that had been stably transfected with full-length mouse CD2 (2B4.CD2; reference 26). T cell antigen recognition by 2B4.CD2 cells, as measured by IL-2 secretion, was substantially enhanced by the expression of CD48 on I-E^{k+} CHO APCs (Fig. 3 A). This enhancement was inhibited by incubating the APCs with a blocking CD48 mAb (Fig. 4 C), and was lost in CD48$^-$ revertants of these APCs (Fig. 3 A). In contrast, the CD48 mAb had no effect on the 2B4 cell response to CD48$^-$

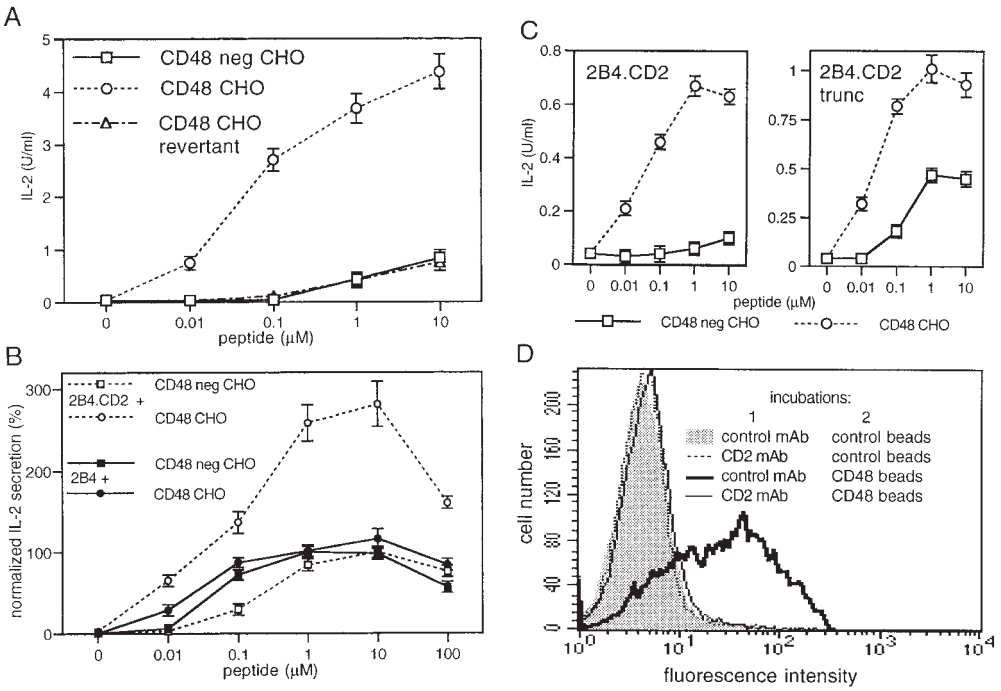

Figure 3. The CD2/CD48 interaction enhances T cell antigen recognition. (A) Antigen recognition by 2B4.CD2 cells using as APCs untransfected I-E^{k+} CHO cells (CD48 neg CHO), I-E^{k+} CHO cells stably transfected with CD48 (CD48 CHO), or $CD48^-$ revertant cells derived from the latter clone (CD48 CHO revertant). In each well, 5×10^4 2B4.CD2 cells were mixed with the same number of APCs and the indicated concentrations of peptide. After 18 h of culture, supernatants were analyzed for IL-2. Error bars represent the SE of triplicate cultures. (B) Comparison of antigen recognition by 2B4 cells expressing high levels (2B4.CD2) or very low levels (2B4) of CD2 using $CD48^+$ or $CD48^-$ I-E^{k+} CHO cells as APCs. Experiment performed as in A. The results were normalized to aid comparison between the different 2B4 cells, letting 100% equal maximal peptide-stimulated IL-2 secretion in the presence of CD48 neg CHOs. (C) Comparison of antigen recognition by 2B4 cells expressing full-length (2B2.CD2) or truncated (2B4.CD2trunc) forms of CD2 cells with $CD48^+$ or $CD48^-$ I-E^{k+} CHO cells as APCs. Experiment performed as in A. (D) Binding of CD48-coated beads to 2B4.CD2 cells is blocked by a CD2 mAb. 2B4.CD2 T cells were incubated with a mouse CD2 (RM2-1) or control (OX11) mAb before incubation with CD5- (control) or CD48-coated fluorescent beads, followed by flow cytometry. Identical results were obtained with 2B4.CD2trunc T cells.

I-E^{k+} CHO cells (Fig. 4 C) nor on the CTLL line used in the IL-2 assay (data not shown), indicating that the inhibitory effect was not the result of binding to 2B4 or CTLL-2 cells (which also express CD48). The CD48 mAb consistently inhibited IL-2 secretion to below the levels seen with $CD48^-$ I-E^{k+} CHO cells (Fig. 4 C). This may be because mAb treatment killed up to 30% of $CD48^+$ I-E^{k+} CHO cells (data not shown).

Recently, the CD2-like 2B4 molecule has been shown to be a second ligand for CD48 (40), raising the question as to whether the CD48 on I-E^{k+} CHO cells was interacting with the 2B4 molecules on 2B4 cells. It was not possible to use a blocking CD2 mAb to test this because ligation of CD2 with mAb appears to transduce an inhibitory signal in 2B4 cells (26). However, two observations indicate that the enhancement by CD48 involves binding to CD2 alone. First, the enhancement was much reduced in 2B4 cells expressing very low levels of CD2 (Fig. 3 B), and second, the binding of CD48-coated beads to 2B4 cells is completely blocked by anti-CD2 mAbs (Fig. 3 D).

Although these results clearly demonstrate that an interaction between CD2 on T cells and CD48 on APCs can substantially enhance T cell antigen recognition, they do not reveal the mechanism of this effect. In addition to being an adhesion molecule, there is evidence that CD2 may transduce signals that activate T cells in the human (55) and the rat (30) or inhibit T cells in the mouse (26). Signaling through CD2 can be abolished by truncation of its cytoplasmic domain (12, 25, 26, 30). We found that CD48 is also able to enhance antigen recognition by 2B4 cells that express a truncated form of mouse CD2 lacking all but 19 of the 116 amino acids normally present in the cytoplasmic domain (Fig. 3 C). Studies using truncated forms of mouse, rat, and human CD2 indicate that this truncation abolishes antibody-induced signaling (12, 25, 26, 30). These results indicate that the enhancing effect of the CD2/CD48 interaction is at least partly a consequence of improved adhesion. Indeed, recent studies (17, 56) suggest that the weaker enhancing effect observed with truncated CD2 may reflect a role for the cytoplasmic domain in enhancing adhesion rather than in transducing a signal.

Long Forms of CD48 Inhibit the T Cell Antigen Recognition. In striking contrast to the effect of wild-type CD48, transfection of I-E^{k+} CHO APCs with long forms of CD48 inhibited T cell antigen recognition (Fig. 4 A). It is notable that the slightly longer form of CD48, CD48-CD22, showed greater inhibition than CD48-CD2 (Fig. 4 A). CD48-CD22 had the larger inhibitory effect in seven out of eight independent antigen response assays (data not shown). The single exception is shown in Fig. 4 D, where CD48-CD2 and CD48-CD22 inhibit to a similar extent. 2B4 cells expressing very low levels of CD2 exhibited only very weak inhibition (Fig. 4 B), consistent with an effect mediated by CD2 ligation. Blocking anti-CD48 antibodies reversed the inhibitory effect of elongated CD48 (Fig. 4 C). This reversal was partial, possibly because the anti-CD48 antibody killed a proportion of the CD48-expressing I-E^{k+} CHO cells (not shown). Finally, the inhibitory effect was completely lost in $CD48^-$ revertants derived from the CD48-CD2– and CD48-CD22–transfected I-E^{k+} CHO cells (Fig. 4 D).

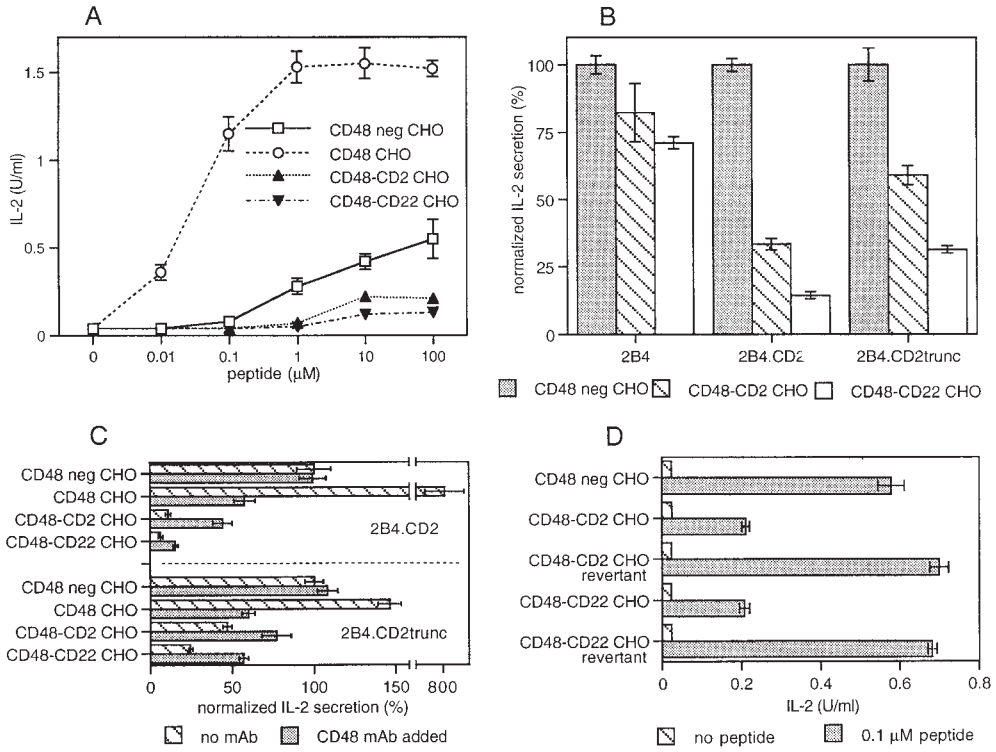

Figure 4. Elongated forms of CD48 inhibit T cell antigen recognition. (A) Antigen recognition by 2B4.CD2 cells using as APCs I-E^{k+} CHO cells expressing no CD48 (CD48 neg CHO), wild-type CD48 (CD48 CHO), CD48-CD2, or CD48-CD22. The assay was performed as described in the legend to Fig. 3 A. The error bars for cultures containing CD48-CD2 and CD48-CD22 I-E^{k+} CHO cells are smaller than the triangular symbols and cannot be seen. (B) CD48-CD2 or CD48-CD22 inhibit antigen recognition by 2B4 cells expressing full-length (2B2.CD2) or truncated (2B4.CD2trunc) CD2. Assay performed as in A with 10 μM peptide. (C) The stimulatory effects of CD48 and inhibitory effects of CD48-CD2 and CD48-CD22 are reversed by a blocking CD48 mAb. Assay performed as in A with 1 μM peptide except that before the assay, the I-E^{k+} CHO cells were incubated for 1 h with 10 μg/ml of the CD48 mAb OX78 and the mAb included throughout the assay. The background response (in the absence of peptide) was subtracted from all values. In B and C, the results were normalized to aid comparison between the different 2B4 cells, letting 100% equal maximal peptide-stimulated IL-2 secretion in the presence of CD48 neg CHOs. (D) Revertant ($CD48^-$) cells derived from the CD48-CD2 and CD48-CD22 I-E^{k+} CHO clones do not inhibit the antigen response of 2B4.CD2trunc cells. Revertant cells expressed the same levels of I-E^k as their parent clones (not shown). Assay performed as in A.

These findings strongly suggest that the inhibitory effect is a consequence of CD2 ligation by the elongated forms of CD48, but do not indicate the mechanism of the inhibition. Since CD2 has been reported to transduce inhibitory signals when cross-linked with mAbs (26), it was important to exclude that inhibitory signaling through CD2. 2B4 cells expressing the truncated form of CD2 (2B4.CD2trunc), which does not transduce an inhibitory signal (26), was also inhibited by expression of elongated CD48 (Fig. 4 B). We also examined whether long forms of CD48 are inhibitory when T cells are activated by mechanisms that do not require peptide–MHC ligation. When 2B4.CD2trunc cells were activated with a phorbol ester plus ionomycin (Fig. 5 A) or an immobilized anti-CD3 mAb (Fig. 5 B), CHO cells expressing long forms of CD48 had no inhibitory effect. Similar results were obtained with 2B4 cells expressing full-length CD2 (not shown). Thus, the inhibitory effect of elongated CD48 was only evident when T cells were activated by TCR engagement of peptide–MHC. Taken together, these results indicate that the inhibitory effect of elongated CD48 is not a consequence of negative signaling through CD2.

A Shortened Form of CD48 Enhances the T Cell Antigen Recognition. We next examined the effect of shortening CD48. Because CD48 has only two Ig domains, it was only possible to shorten it by a single domain (~3.5 nm). In contrast to the long forms of CD48, transfection of I-E^{k+} CHO with a shortened form of CD48 (CD48d1; Fig. 1 A) enhanced peptide–MHC-induced activation of 2B4 (Fig. 6). Enhancement by CD48d1 was observed with 2B4 cells expressing both full-length (Fig. 6 A) and truncated (Fig. 6 B) CD2, but was somewhat reduced compared with wild-type CD48 (Fig. 6). Because the surface expression of CD48d1 was only half that of CD48 (Fig. 2 B), and CD2 binding to these cells was also substantially reduced (Fig. 2 B), it is not possible to deduce from these data how much less effective CD48d1 is than full-length CD48 at enhancing T cell antigen recognition. Nevertheless, it is clear that shortening of CD48 does not have the same inhibitory effect as elongation. This is an important control, since both shortening and elongation of CD48 would be expected to disrupt *cis*-interactions with other surface molecules. It follows that elongation of CD48 cannot be inhibiting by disrupting such *cis*-interactions.

Long Forms of CD48 Inhibit TCR Downmodulation. To further investigate the mechanism by which long forms of CD48 inhibited T cell antigen recognition, we investigated the effect of elongated CD48 on TCR downmodulation. Recent studies suggest that TCR downmodulation is a consequence of TCR engagement (57, 58), and is relatively independent of costimulation and downstream signaling events. Although wild-type CD48 enhanced TCR downmodulation induced by peptide-loaded I-E^{k+} CHO cells, CD48-CD22 strongly inhibited downmodulation (Fig. 7). This sug-

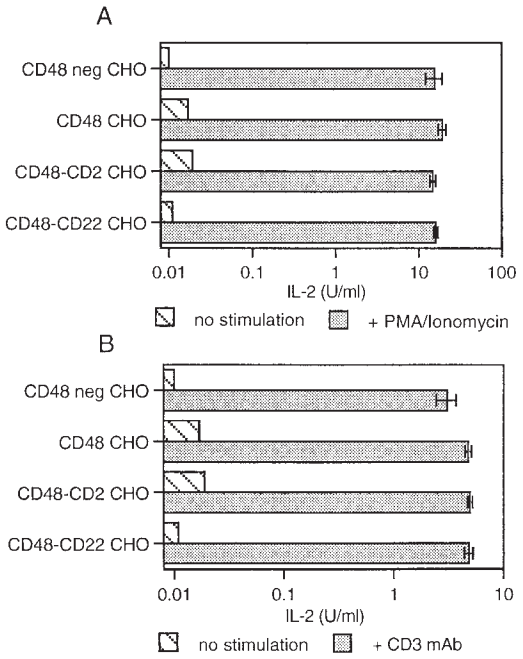

Figure 5. Elongated CD48 molecules do not inhibit the response of T cells to antigen-independent stimuli. (A) CHO cells with elongated CD48 do not inhibit T cell activation by PMA/ionomycin. Assay performed as in the legend to Fig. 3 A except that, instead of peptide antigen, PMA (50 ng/ml) plus ionomycin (500 ng/ml) was used to stimulate 2B4.CD2trunc T cells. (B) CHO cells with elongated CD48 do not inhibit T cell activation by CD3 mAb. Assay performed as in the legend to Fig. 3 A except that, instead of peptide antigen, CD3 mAb 145-2C11 was immobilized to microtiter plates (see Materials and Methods) before addition of 2B4.CD2trunc T cells and APCs. Similar results were obtained when soluble 145-2C11 was used to stimulate the T cells (not shown).

gests that wild-type CD48 enhances, whereas elongated CD48 inhibits, TCR engagement of peptide–MHC.

Inhibition of TCR/Peptide–MHC Interaction by Elongation of the CD2/CD48 Complex. The key observation in this study is that whereas expression of wild-type CD48 on APCs enhances T cell antigen recognition, CD48 elongated by two or three Ig-like domains is strongly inhibitory. The most likely explanation for these results, strongly

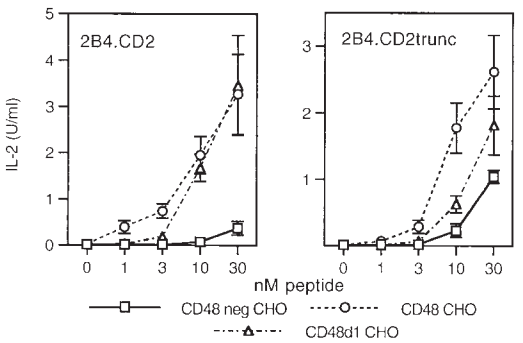

Figure 6. A shortened form of CD48 is able to enhance T cell antigen recognition. Assay performed as in the legend to Fig. 3 A using as responders 2B4 cells expressing either full-length (2B4.CD2) or truncated (2B4.CD2trunc) forms of CD2, and as APCs I-E^{k+} CHO cells expressing no CD48 (CD48 neg CHO), wild-type CD48, or shortened CD48 (CD48d1 CHO).

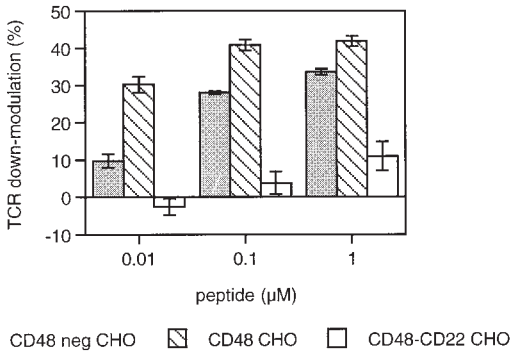

Figure 7. Elongated CD48 inhibits TCR downmodulation. TCR downmodulation was measured by flow cytometry on 2B4.CD2 cells after coculture with the indicated I-E^{k+} CHO cell APCs loaded with various concentrations of MCC peptide. Downmodulation is expressed as the percent reduction in mean TCR levels, with 0% downmodulation equal to the TCR level measured in the absence of peptide. The values shown are the mean ± SD of triplicate cultures. Data shown are representative of three independent experiments.

supported by measurements of TCR downmodulation (Fig. 7), is that binding of CD2 on T cells to elongated forms of CD48 on APCs positions the plasma membranes at a distance too far apart for efficient TCR engagement of peptide–MHC (Fig. 8, right). Conversely, the binding of CD2 to wild-type CD48 promotes TCR/peptide–MHC engagement through a favorable positioning of the plasma membranes (Fig. 8, left). Control experiments have excluded several other explanations. Thus, the inhibitory effect is not the result of negative signaling through CD2, nor is it likely

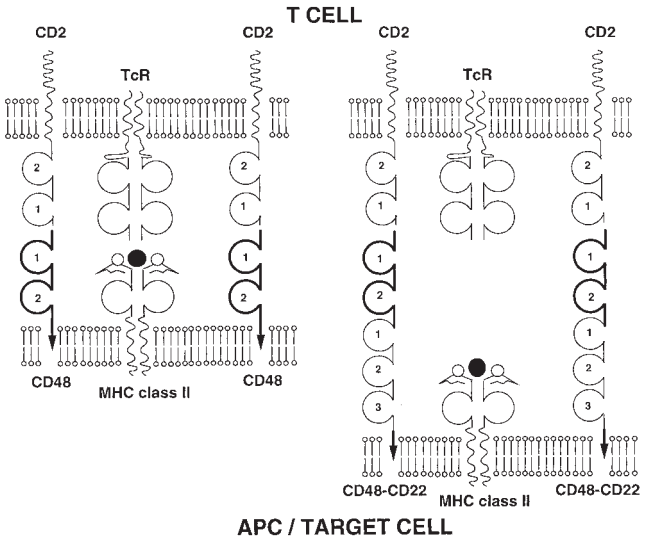

Figure 8. A model explaining the contrasting effects of wild-type and elongated CD48 on T cell antigen recognition. CD2 molecules on T cells and CD48 molecules on I-E^{k+} CHO APCs interact to form contacts in which the intermembrane separation distance is determined by the dimensions of the CD2/CD48 complex. Wild-type CD48 (left) enhances T cell antigen recognition because the separation distance (~15 nm) is optimal for TCR engagement of peptide–MHC. Elongated CD48 (CD48-CD22, right) inhibits T cell antigen recognition by forming contacts in which the intermembrane distance (>20 nm) is too great for TCR to engage peptide–MHC.

to be a consequence of the disruption of *cis*-interactions between CD48 and other molecules. Although our data indicate that TCR/peptide–MHC engagement is inhibited, we cannot formally exclude the possibility that elongated forms of CD48 also inhibit T cell antigen recognition by forcing the segregation of CD2 from the TCR, thereby sequestering CD2-associated molecules required for TCR signaling.

CD2 and the "Kinetic-segregation" Model of T Cell Antigen Recognition. The human CD2 fragment inserted into the CD48-CD2 chimera is ~7 nm long (15). Since the length of the CD2/CD48 complex is ~14 nm (8), the CD2/CD48-CD2 complex will span 14–21 nm. If the proposed explanation for the inhibitory effects of elongated CD48 is correct (Fig. 8), our data imply that optimal TCR engagement of peptide–MHC requires that the plasma membranes of the T cell and APC have to be <21 nm apart. It follows that molecules larger than this (e.g., CD45 and CD43), and molecular interactions that span greater distances (e.g., LFA-1/intercellular adhesion molecule [ICAM]-1), would need to be excluded from the immediate vicinity of the TCR when it engages peptide–MHC.

Our results thus provide support for a model proposed by Davis and van der Merwe (henceforth called the "kinetic-segregation" model) for T cell antigen recognition (13). This model postulates that molecules in the contact area between T cells and APC/target cells will segregate according to size, and that this segregation is a prerequisite for TCR triggering. The concept of segregation of T cell surface molecules according to size was first introduced by Springer (10). A similar model has been also been proposed recently by Shaw and Dustin (9). The kinetic-segregation model postulates that small molecules such as CD2, CD4, CD8, CD28, and CD154 will localize, together with their extracellular and cytoplasmic ligands, in zones of especially intimate contact (~15 nm between membranes) called "close-contact zones," and that larger molecules such as integrins, CD43, and CD45 would be excluded from these zones. It is proposed that TCR binding to peptide–MHC leads to TCR triggering by trapping the TCR/CD3 complex within the tyrosine kinase–enriched (and tyrosine phosphatase–deficient) close-contact zone for a period of time sufficient for tyrosine phosphorylation of TCR/CD3-associated signaling molecules. Several lines of evidence support this model. First, intermembrane distances of 12–15 nm have been measured in the contact area between cytotoxic T cells and target cells (59). Second, Kupfer and colleagues (60) have shown that TCR/CD3 and the integrin LFA-1 are segregated into different regions at the T cell/APC interface. Third, Dustin et al. (17) have shown that when Jurkat cells are allowed to adhere to lipid-anchored CD48 and ICAM-1 (CD54, an LFA-1 ligand) inserted into glass-supported planar lipid bilayers, the ICAM-1 and CD48 segregate from each other in the contact area. Fourth, Sperling et al. (61) have reported that CD43 is completely excluded from the binding interface between T cells and APCs. Interestingly, CD45 was not excluded from the binding interface, and was sometimes polarized towards it (61). Higher resolution imaging studies will be required to determine whether, like LFA-1 (17, 60), CD45 segregates from the TCR within the interface. Finally, our finding that elongation of CD2/CD48 complexes to the approximate size of an integrin molecule (~21 nm) inhibits T cell antigen recognition provides experimental support for the notion that segregation is a prerequisite for TCR engagement of peptide–MHC.

Our findings raise the question as to why large cell–cell adhesion molecules such as the integrin LFA-1 do not inhibit T cell antigen recognition through the same mechanism as postulated here for the elongated forms of CD48. Indeed, LFA-1 interactions appear to enhance TCR engagement of peptide–MHC (62). One possible explanation, strongly supported by recent data (60), is that T cells have evolved specific mechanisms, perhaps cytoskeletally driven (63), to actively segregate LFA-1 from the TCR within the contact zone. This would enable LFA-1 to participate in T cell–APC conjugate formation without physically interfering with TCR binding to peptide–MHC. In contrast, there is evidence that CD2 may be actively clustered within the contact zone into the immediate vicinity of the TCR (17). This would be expected to prevent the segregation of elongated CD2/CD48 complexes from the site of TCR engagement.

Since CD48d1 is missing a single Ig domain (~3.5 nm), it can reasonably be predicted that the CD2/CD48d1 complex will be ~3.5 nm shorter than the CD2/CD48 complex (i.e., ~10.5 nm). Our finding that CD48d1 can still enhance T cell antigen recognition implies that some reduction in the size of accessory molecules (and thus intermembrane distance) can be tolerated. Mechanisms by which the TCR/peptide–MHC interaction could adjust to closer membrane approximation include compression of long stalk regions of the TCR, which lack secondary structure, or tilting of the TCR/peptide–MHC complex relative to the plane of the membrane. Some limited flexibility in the size of accessory molecule and TCR/peptide–MHC complexes may be important because it is unlikely that all molecular interactions involved in T cell antigen recognition will span exactly the same distance.

The kinetic-segregation model postulates that small accessory molecules contribute to T cell antigen recognition in part by driving the formation of intimate close-contact zones. While the CD2/ligand interaction may contribute to the formation of close-contact zones, any accessory molecule interaction with approximately the same dimensions could fulfill the same function. This is supported by the observation that CD2-deficient mice have very mild abnormalities in T cell function (27, 29). Candidate accessory molecules include CD28/ligand interactions and interactions involving other members of the CD2 family. The domain composition of CD28 and its ligands CD80 and CD86 suggests that these interactions will span 10–15 nm (7). Although CD28 can transduce activation signals through its cytoplasmic region (64), the recent observation that a form of CD28 lacking a cytoplasmic domain is very effective at enhancing T cell antigen recognition (65) provides strong support for an important adhesion role. Structural and mutagenesis studies suggest that interactions between other CD2 family members will span the same distance as the

CD2/CD48 complex (13, 46), making them suitable for membrane approximation in T cell antigen recognition. Potential candidates include SLAM (66), which is expressed on T cells, and 2B4, which is expressed on NK cells and a subpopulation of T cells (67, 68), and has recently been shown to bind CD48 (40).

In conclusion, our results demonstrate the importance of accessory molecule size, and suggest that close membrane approximation is required for T cell antigen recognition. This and other recent studies (17, 60, 61) support models of T cell antigen recognition (9, 13) which propose that the cell surface molecules segregate according to size at the T cell/APC interface, and that this segregation is required for TCR engagement and triggering.

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