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Immunology

Physical association of CD4 and the T-cell receptor can be induced by anti-T-cell receptor antibodies

(T-cell activation/human immunodeficiency virus)

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ABSTRACT **CD4 can physically associate with the CD3–T-cell receptor complex as visualized in cocapping experiments. This association occurs when the T-cell receptor is cross-linked by certain anti-variable region antibodies that appear to induce a conformational change in the receptor such that it associates with CD4. Similar association has been observed in earlier studies with the same cloned helper T cell when the physiological ligand, antigen–class II major histocompatibility complex molecule, is bound by the T-cell receptor. The ability of anti-T-cell receptor antibodies to induce the T-cell receptor–CD4 association correlates with a 100-fold increase in the ability of the antibody to activate the T cell. This suggests that the complex of CD4 and the T-cell receptor act synergistically in T-cell activation, thus readily explaining the commonly observed association of CD4 expression with class II major histocompatibility complex-restricted antigen recognition. This association could also play a role in infection by human immunodeficiency virus.**

T lymphocytes recognize antigen as peptide fragments bound to molecules encoded in the major histocompatibility complex (MHC) (1). There are two distinct classes of MHC glycoproteins that subserve this antigen-presenting function, called class I and class II MHC molecules. The T cells responding to antigen presented by these two classes of MHC molecule can also be distinguished by means of differential expression of cell surface molecules (2). T cells bearing CD4 molecules recognize peptide fragments bound to class II MHC molecules, while T cells bearing CD8 molecules recognize peptide fragments bound to class I MHC molecules.

The T-cell receptor recognizes not only the specific foreign antigen but also the MHC molecule that presents it. Recognition of both antigen and MHC molecule is mediated by the highly variable α and β chains of the T-cell receptor (3, 4). Although CD4 is associated with class II MHC recognition by T cells, it has been considered to be an accessory molecule in T-cell antigen–class II MHC recognition for several reasons. First, CD4 is not variable (5), while T-cell recognition of the highly polymorphic class II MHC molecules is exquisitely precise (6). Second, when the T-cell receptor is modulated off the T-cell surface by anti-T-cell receptor antibody, CD4 expression is reported to be unaltered (7). Third, although CD4 is judged not to be part of the T-cell receptor, anti-CD4 often inhibits responses of CD4-bearing T cells to antigen–class II MHC ligands (2, 8, 9). This effect has most commonly been attributed to an adhesion-strengthening function of CD4 (10). Indeed, CD4 can bind class II MHC molecules directly (11).

Recently, we have obtained data that suggest that CD4 is actually a physical component of the T-cell receptor for antigen–class II MHC. Antibodies to the T-cell receptor will activate CD4$^+$ T cells (12); while anti-CD4 does not usually affect these responses (9), it was found that anti-CD4 does inhibit responses induced by anti-T-cell receptor antibodies directed at a particular variable (V) region epitope (13). Second, T cells bearing CD4 and CD8 but responding to class II MHC molecules are 100-fold more susceptible to inhibition by anti-CD4 than to inhibition by anti-CD8, although ligands for both CD4 (class II MHC) and CD8 (class I MHC) are present on the stimulating cell (14, 15). Third, CD4 and the T-cell receptor cluster at the site of antigen–class II MHC recognition (16). Finally, modulation of the T-cell receptor with certain anti-T-cell receptor antibodies does comodulate CD4 (17). These and other functional studies suggest that CD4 is actually a part of the T-cell receptor for antigen–class II MHC (18–20). However, direct evidence for such a physical association is lacking. In the present experiments, we demonstrate that CD4 and the T-cell receptor rapidly colocalize on the T-cell surface under particular experimental conditions. These experimental conditions coincide with those for the most effective T-cell activation, are specific for CD4 and the T-cell receptor, and occur only in one direction. These observations strongly support the role of CD4 as an important component of the T-cell receptor for antigen–class II MHC.

MATERIALS AND METHODS

Cells. The cloned L3T4$^+$ helper T-cell line D10.G4.1 (D10) was used. D10 cells are specific for hen egg conalbumin in the context of I-A^k molecules. The cells were maintained in culture as described in detail previously (12).

Antibodies. Mouse monoclonal antibodies specific for the T-cell receptor of D10 were 3D3, 10B, 4B, 5A (all IgG1), and 16A (IgG3) (21). The mouse anti-cell receptor antibody F23.1 (IgG2a) (22) and 145-2C11, an Armenian hamster monoclonal antibody specific for mouse CD3ε chains (23), were also used. Other monoclonal antibodies used were rat antibodies specific for mouse L3T4 (GK1.5) (24, 25) and for LFA-1 (M17/5.2) (26). All antibodies were purified by affinity chromatography over staphylococcal protein-A columns (MAPS II, Bio-Rad). Samples of the antibodies were biotinylated and used at optimal concentrations for staining as determined by fluorescence-activated cell sorting after incubation with fluorescein-avidin (Vector Laboratories).

For indirect staining using nonbiotinylated antibodies, affinity-purified fluorescein isothiocyanate (FITC) conjugates of goat anti-mouse IgGs (Hyclone), were used. These were found to cross-react extensively with both rat and Armenian hamster IgGs. Reactivity to rat or Armenian hamster immunoglobulins was selectively removed by pas-

Abbreviations: MHC, major histocompatibility complex; V, variable; FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate.

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sage through columns of protein A-purified normal serum IgG from rats or Armenian hamsters coupled to Sepharose 4B (Pharmacia). FITC-conjugated goat anti-rat IgG (mouse-adsorbed, Hyclone) was also used.

Capping Induction and Immunofluorescence Staining. For induction of capping, cells (5×10^5) were incubated with antibodies against T-cell receptor, CD3, LFA-1, or L3T4 for 1 hr at 37°C in Click's medium containing 10% fetal calf serum, washed, and incubated for 30 min at 37°C with unadsorbed FITC-conjugated goat anti-mouse IgG. The cells were then washed three times with Dulbecco's phosphate-buffered saline containing 0.1% sodium azide and fixed with 1% paraformaldehyde in saline, pH 7.4. Some of the anti-D10 T-cell receptor antibodies actively induced capping of the T-cell receptor (see *Results*). In these cases, the incubation with the fluorescent anti-mouse immunoglobulin was performed at 4°C in phosphate-buffered saline containing 2% fetal calf serum and 0.1% sodium azide (staining buffer).

For double staining of the cells, incubation with FITC-conjugated goat anti-mouse immunoglobulin or FITC-conjugated goat anti-rat immunoglobulin was followed by washing in staining buffer and addition of biotinylated antibody against another surface molecule. In this case, the FITC-coupled antibodies were previously adsorbed with immunoglobulins of the species to which the biotinylated antibodies belonged. After a 30-min incubation at 4°C, the cells were washed again, incubated with rhodamine isothiocyanate (RITC)-avidin (Vector Laboratories) in staining buffer (30 min at 4°C), washed in phosphate-buffered saline/sodium azide, and fixed at 1% paraformaldehyde. The cells were examined under appropriate fluorescence or transmitted light with an Olympus BHTU microscope.

RESULTS

These experiments were carried out using one well-characterized CD4$^+$ T-cell line with potent helper function, D10.G4.1 (D10) (12). We have produced several monoclonal antibodies specific for the α/β heterodimeric T-cell receptor on this cloned T-cell line. As reported previously, these antibodies are directed at distinct V region epitopes on the receptor of the D10 cloned T-cell line (21). The ability of these antibodies to activate this cloned T-cell line varies by over 100-fold per molecule of bivalent anti-receptor antibody bound. Some very low affinity antibodies are of high potency, providing they bind epitopes I or I′. These antibodies, plus antibodies to CD3, CD4, and LFA-1, were used for staining (Table 1).

The 3D3 Anti-D10 T-Cell Receptor Antibody Partially Cocaps CD4. To determine whether CD4 can physically associate with the T-cell receptor on cloned T-cell line D10, the T-cell receptor was capped with 3D3, an anticlonotypic anti-D10 T-cell receptor antibody. The capping was revealed by using FITC-coupled anti-mouse immunoglobulin, as shown in Fig. 1*a*. The distribution of CD4 was then examined by using biotinylated rat anti-CD4 monoclonal antibody and RITC-avidin. As can be seen in Fig. 1*b*, this anti-T-cell receptor antibody cocaps most of the surface CD4. Some CD4 does not cocap with the T-cell receptor, and this is probably due to the fact that CD4 is 2–3 times more abundant than the T-cell receptor on the surface of D10 cells (17, 18). By contrast, another molecule involved in T-cell–antigen-presenting cell interactions, LFA-1, also detected by biotinylated rat monoclonal antibodies, does not cocap with the T-cell receptor when capping is induced by 3D3 (Fig. 1 d and e).

Anti-CD3 and the T-Cell Receptor Cocap, but Anti-CD3 Does Not Cocap CD4. Like the anti-T-cell receptor V region antibodies tested above, anti-CD3 also activates D10 cells (21). On D10 cells, where CD3 and the α/β heterodimer of the T-cell receptor can be visualized, they cocap completely (Fig. 1 *g*, *h*). This is consistent with previous results using human T cells (7). However, anti-CD3 does not cocap CD4 (Fig. 1 *m*, *n*). Thus, CD3 and the T-cell receptor are stoichiometrically and stably associated on all T-cell lines, but the CD3–T cell receptor complex is not stably associated with CD4.

Table 1. Induction of capping by antibodies against D10 T-cell receptor–CD3 complex

Antibody*	Specificity†	Activating potency‡	% capping§
3D3	Clonotypic (I)	High	46.4
10B	Clonotypic (I)	High	49.4
4B	Clonotypic (I′)	High	57.4
16A	Clonotypic (II)	Low	17.7
5A	Clonotypic (III)	Low	23.2
F23.1	T-cell receptor, $V_\beta 8$ subfamily (V)	Low	18.3
145-2C11	CD3ε	ND	11.2

*Antibodies 3D3, 4B, 5A, 16A, and F23.1 recognize different epitopes in the D10 T-cell receptor. Antibody 10B binds to an epitope similar to 3D3 (21).

†As determined in ref. 21 for the clonotypic antibodies, ref. 27 for F23.1, and ref. 23 for 145-2C11. In parentheses, epitope bound as defined in ref. 21.

‡Potency is defined as the ratio of antibody concentration inducing 50% maximal activation of D10 proliferation to K_d, as described in ref. 21. ND, not determined.

§Percentage of D10 cells showing T-cell receptor capping after incubation for 1 hr at 37°C in the presence of anti-T-cell receptor or anti-CD3 antibodies. Capping was revealed by further incubation with FITC-conjugated goat anti-mouse immunoglobulin for 30 min at 4°C in staining buffer. The percentage was calculated from a minimum of 200 cells. The percentage of cells showing capping after incubation with 3D3 at 4°C in staining buffer followed by FITC-conjugated goat anti-mouse immunoglobulin staining at 4°C was 9.6.

Capping of CD4 Does Not Cocap the T-Cell Receptor Complex. Anti-CD4 does not cause capping of CD4 unless anti-rat immunoglobulin is added to cross-link the anti-CD4. When this is done, CD4 is capped, as shown in Fig. 1*j*. When cells capped with anti-CD4 are stained with anti-CD3 or anti-T-cell receptor antibodies, then no cocapping is observed (Fig. 1*k* and data not shown). Thus, CD4 is not stably associated with readily detectable amounts of CD3–T-cell receptor complexes.

High-Potency Anti-T-Cell Receptor Antibodies Cocap CD4, While Low-Potency Anti-T-Cell Receptor Antibodies Do Not. In a previous publication, we have shown striking differences between anti-D10 T-cell receptor monoclonal antibodies in terms of the number of molecules of antibody bound required to induce half-maximal proliferation or lymphokine secretion by this cell line, although all antibodies tested achieve maximal activation at concentrations lower than those used in capping experiments. The "potency" of the antibodies correlates with the particular epitope recognized (Table 1), rather than with affinity or other characteristics (21). Since 3D3 is an antibody of high activating potency, we have examined a panel of anti-D10 T-cell receptor antibodies of various potencies to determine their ability to induce cocapping of CD4. Two results are of interest. First, only high-potency antibodies (10B, 4B) induce cocapping of CD4 with the T-cell receptor as previously shown for 3D3 (Fig. 2 *a*, *b*; and *d*, *e*). Low-potency antibodies directed at three distinct epitopes (5A, 16A, F23.1) do not cocap CD4 (Fig. 2 *g*, *h*; *j*, *k*; and *m*, *n*). This is in agreement with previous data obtained with D10 showing that F23.1 did not induce cocapping of CD4 with the T-cell receptor (16). The second observation concerns the ability of the anti-T-cell receptor antibodies to induce capping of the T-cell receptor directly. Potent antibodies such as 3D3, 10B, or 4B efficiently induce capping of the T-cell receptor on D10 cells after incubation for 1 hr at 37°C, while low-potency antibodies (5A, 16A, F23.1) did so

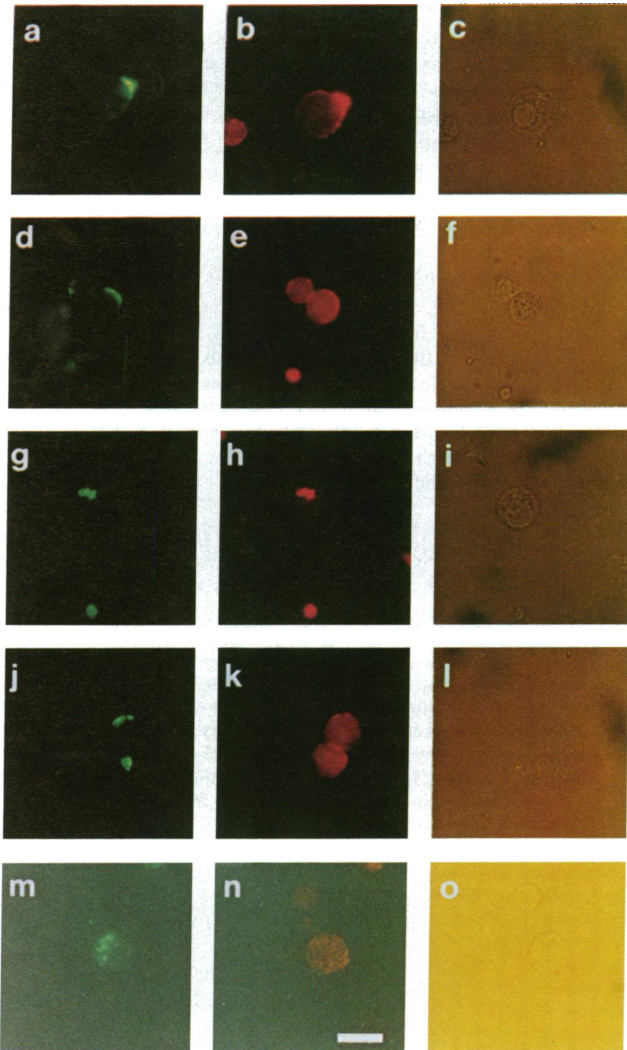

FIG. 1. Capping of D10 T-cell receptor by anti-clonotypic antibody 3D3 (*a*, *d*, *g*); or capping of CD4 by GK1.5 (*j*) or CD3 by 145-2C11 (*m*) was induced and revealed with appropriately adsorbed FITC-conjugated goat anti-mouse or anti-rat immunoglobulin antibodies. Then the capped, fixed cells were incubated with biotinylated antibodies against CD4 (GK1.5; *b*, *n*), LFA-1 (M17/5; *e*), CD3 (145-2C11; *h*) or D10 T-cell receptor (3D3; *k*) and revealed with RITC-avidin. The same fields are also shown under transmitted light (*c*, *f*, *i*, *l*, *o*).

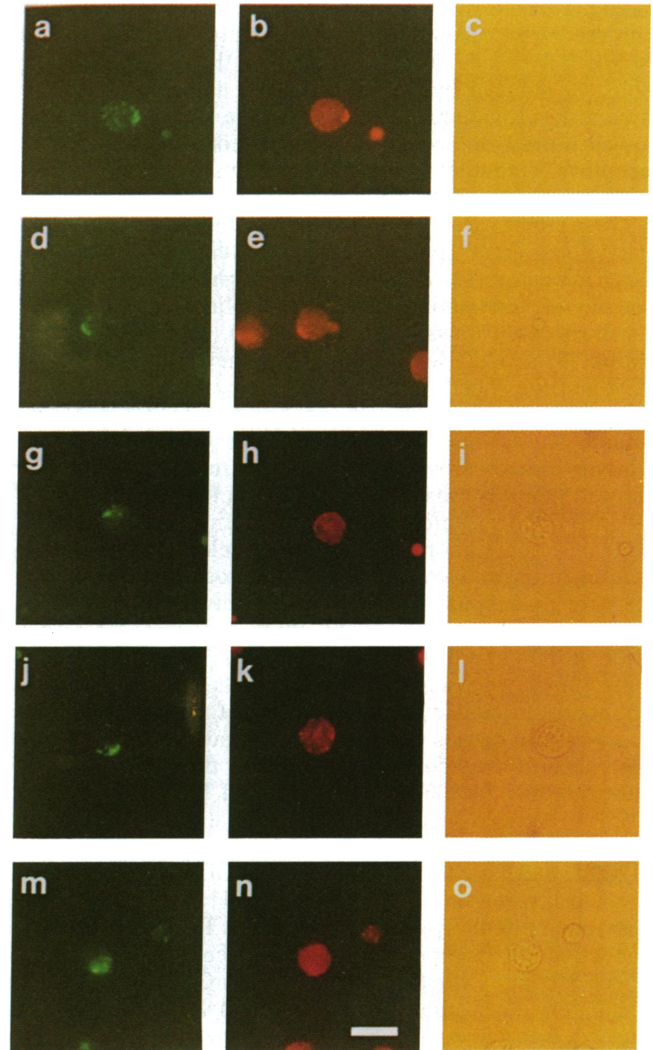

FIG. 2. Capping of the T-cell receptor of D10 was induced by anti-clonotypic antibodies 10B (*a*), 4B (*d*), 5A (*g*), 16A (*j*), or by anti-$V_{\beta}8$ antibody F23.1 (*m*), and revealed by using rat immunoglobulin-adsorbed, FITC-conjugated goat anti-mouse immunoglobulin antibodies. Distribution of CD4 in the same cells was revealed with biotinylated GK1.5 anti-CD4 antibodies and RITC-avidin (*b*, *e*, *h*, *k*, *n*). The same fields are shown under transmitted light in *c*, *f*, *i*, *l*, and *o*. Capping was carried out at 37°C for the first and second steps.

to a much lesser extent (Table 1), making incubation at 37°C with the secondary, FITC-labeled antibody necessary to induce T-cell receptor capping on most cells. The temperature of the second-step incubation did not affect results with high-potency antibodies. These results correlate well with previous data from this laboratory showing that only high-potency anti-D10 cell receptor antibodies such as 3D3, and not low-potency antibodies such as 5A, could modulate the T-cell receptor from the surface of the cells and comodulate CD4 (17).

DISCUSSION

The purpose of this study was to determine whether CD4 associates physically with the T-cell receptor. Indeed, such as physical association was observed when the D10 T-cell receptor was capped by using high-potency anti-T-cell receptor antibodies. However, CD4 and the T-cell receptor are not normally associated on the T-cell surface, as shown by the failure of CD3 and CD4 to cocap, by the failure of anti-CD4 capping to cause cocapping of the CD3–T-cell receptor complex, and by the failure of low-potency anti-T-cell receptor antibodies to cocap the T-cell receptor and CD4. The association of the T-cell receptor and CD4 is specific, since other surface molecules such as LFA-1 do not cocap. Finally, that different anti-T-cell receptor antibodies of the same isotype (3D3 and 5A) and low or high affinity (4B and 5A) (21) do or do not cocap CD4 assures us that cocapping is not due to artifacts in the procedure or differences in affinity of the anti-T-cell receptor antibodies.

These data demonstrate that the physical association of CD4 and the T-cell receptor is dependent on the state of the T-cell receptor. All of the anti-T-cell receptor antibodies tested can maximally activate D10 at the concentrations used in the first step of the capping process (21). Thus, the differences seen in the distribution of CD4 are unlikely to reflect differences in activation of the T cells. Furthermore, these changes occur rapidly, involve only some of the CD4 molecules, and always show precise colocalization with the T-cell receptor. That it is precisely those anti-receptor antibodies previously shown to be of high potency in stim-

ulating D10 cells that also cause physical association of CD4 with the T-cell receptor strongly suggests that the physical association results from a change in the T-cell receptor molecule induced by the anti-receptor antibody. We have previously provided evidence that these high-potency antibodies indeed do induce a conformational change in the receptor (21) that leads to a biologically significant physical association with CD4 (17).

These studies are also consistent with the notion that T-cell activation is greatly potentiated by cross-linking CD4 and the T-cell receptor (19, 28). Thus, one might explain the high potency of some antibodies by their ability to cross-link the T-cell receptor in association with CD4, while low-potency antibodies activate less well because they fail to colocalize CD4 and the T-cell receptor. Studies by Eichmann, Emmrich, *et al.* (28, 29) and by Owens, Fazekas de St. Groth, and Miller (30, 31) have all supported this hypothesis. These workers directly cross-linked the T-cell receptor to CD4 and showed that this cross-linking greatly potentiated activation by anti-T-cell receptor antibodies. Taken together with our earlier demonstrations of CD4 and T-cell receptor colocalization during antigen–class II MHC recognition, these studies strongly support the idea that it is this positive signaling aspect of CD4 function (13, 17, 18) that explains the association of CD4 expression and class II MHC recognition by T cells (2). If one assumes that it is the ability of some antibodies to induce the association of CD4 with the T-cell receptor that causes the differences in potency we observe, then ligands that colocalize CD4 and the T-cell receptor should reduce by 100-fold the amount of T-cell receptor that needs to be cross-linked to achieve activation. Assuming that it is binding of CD4 and the T-cell receptor to the same class II MHC molecule that normally drives CD4 association with the T-cell receptor (11, 16), then a $CD4^+$ T cell will require only one 1/100th as many antigen–class II MHC complexes for activation as compared to a $CD4^-$ T cell bearing the same T-cell receptor, whether or not it bears CD8. We assume a similar effect will apply to CD8 and class I MHC recognition. Indeed, transfection of CD8 to class I-specific, $CD8^-$ T cells reduces the antigen dose required for T-cell activation by about 100-fold (32, 33).

Thus, the conditional physical association of CD4 with the T-cell receptor, demonstrated here by cocapping and previously by comodulation (17) and by biological assays (13, 17, 18), can readily account for the strict association of CD4 expression and class II MHC-restricted antigen recognition. Similar conclusions were recently reached in studies using human T cells as well (34). Earlier hypotheses of adhesion strengthening or increased affinity mediated by CD4 binding to class II MHC molecule cannot explain this specificity association. This follows from the consideration that antigen-presenting cells expressing class II MHC molecules also express class I MHC molecules. If CD4 binds class II MHC molecules while CD8 binds class I MHC molecules, an antigen-presenting cell bearing a class II MHC-stimulating ligand and expressing both class I and class II MHC molecules should stimulate $CD4^+$ and $CD8^+$ T cells equivalently unless CD4 and CD8 function as part of the receptor.

Parallel studies of D10 T-cell receptor and CD4 mobility have been carried out concurrently by Kupfer and Singer (35), who compared CD4 cocapping by 3D3 and F23.1. Using different techniques, these authors obtained essentially similar results. In contrast to these authors, we would emphasize that all anti-T-cell receptor antibodies tested to date activate D10 cells in soluble, bivalent form, but only high-potency antibodies induce capping of the T-cell receptor and cocapping of CD4 (Fig. 1 and 2). Thus, in our view, the induction of this physical association is biologically significant, a conclusion shared by Kupfer and Singer.

Finally, these results may have interesting implications for infection by human immunodeficiency virus (HIV). HIV binds to $CD4^+$ T cells via its gp120 surface antigen (36). gp120 binds to CD4 with high affinity (37). To replicate, HIV-infected lymphocytes must be activated (38), while cross-linking CD4 transduces a negative signal for T-cell activation (39–41). However, if the gp120 molecule could cross-link CD4 and the T-cell receptor on certain T cells, it might activate such cells and thus induce a productive infection.

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