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Non-CD28 Costimulatory Molecules Present in T Cell Rafts Induce T Cell Costimulation by Enhancing the Association of TCR with Rafts

Yumi Yashiro-Ohtani,* Xu-Yu Zhou,* Kazuhiro Toyo-oka,* Xu-Guang Tai,* Cheung-Seog Park,* Toshiyuki Hamaoka,* Ryo Abe,† Kensuke Miyake,‡ and Hiromi Fujiwara*2

While CD28 functions as the major T cell costimulatory receptor, a number of other T cell molecules have also been described to induce T cell costimulation. Here, we investigated the mechanisms by which costimulatory molecules other than CD28 contribute to T cell activation. Non-CD28 costimulatory molecules such as CD5, CD9, CD2, and CD44 were present in the detergent-insoluble glycolipid-enriched (DIG) fraction/raft of the T cell surface, which is rich in TCR signaling molecules and generates a TCR signal upon recruitment of the TCR complex. Compared with CD3 ligation, coligation of CD3 and CD5 as an example of DIG-resident costimulatory molecules led to an enhanced association of CD3 and DIG. Such a DIG redistribution markedly up-regulated TCR signaling as observed by ZAP-70/LAT activation and Ca2+ influx. Disruption of DIG structure using an agent capable of altering cholesterol organization potently diminished Ca2+ mobilization induced by the coligation of CD3 and CD5. This was associated with the inhibition of the redistribution of DIG although the association of CD3 and CD5 was not affected. Thus, the DIG-resident costimulatory molecules exert their costimulatory effects by contributing to an enhanced association of TCR/CD3 and DIG. The Journal of Immunology, 2000, 164: 1251–1259.

The activation of resting T cells requires two independent signals (1–4). The first signal stems from the recognition by the TCR of processed Ag peptides plus MHC molecules on APC. The second signal is provided by other receptor-ligand interactions between T cells and APC. Intensive research in the last several years has characterized the T cell molecule CD28 as the receptor that generates costimulatory signals in T cells (2–4); CD28 engagement, by either anti-CD28 mAb or ligands, has been shown to costimulate T cells, resulting in T cell activation (5–7).

In addition to CD28, multiple molecules on the T cell have been shown to costimulate resting T cells, including CD5 (8), CD2 (9), CD44 (10), and CD9 (11); in the presence of suboptimal doses of anti-CD3, each of the mAbs against these molecules costimulated resting T cells as potently as anti-CD28 mAb (11, 12). Regarding the mechanism of CD28 signaling, several reports have suggested that CD28 delivers a signal different from that derived from TCR. Namely, CD28-mediated signaling involves the activation of phosphatidylinositol-3-phosphate kinase (13–15) and, in conjunction with a TCR signal, activates c-Jun NH2-terminal kinase (16, 17), which regulates AP-1 and related transcription factors. Along with the generation of these signals, it is also possible that CD28 functions to enhance TCR-mediated signaling. In contrast, the mechanism by which non-CD28 costimulatory molecules contribute to T cell activation has been poorly investigated.

A recent study demonstrated that T cell activation induced by anti-CD3 stimulation leads to a redistribution of the detergent-insoluble glycolipid-enriched membrane fraction (DIG)3 (also called raft) (18, 19). The raft redistribution results in the association of TCR/CD3 and DIG-associated signal-transducing molecules, indicating the role of such a membrane compartmentation in efficient T cell activation (18, 19). Most recently, Viola et al. (20) showed that CD28 costimulation enhances raft redistribution to the site of TCR engagement. Here, we show that, unlike CD28, non-CD28 costimulatory molecules such as CD5 and CD9 are present in the DIG fraction and that these DIG-resident costimulatory molecules, when coligated with CD3, strengthen TCR-mediated signaling through inducing aggregation of DIG as well as enhanced association of TCR/CD3 and DIG. These results are consistent with the notion that recruitment of TCR/CD3 to DIG is important for enhanced generation of TCR signals and provide a mechanism by which DIG-resident costimulatory molecules exhibit their costimulatory capacity.

Materials and Methods

Mice

C57BL/6 mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and used at 7–9 wk of age.

Reagents

Anti-CD3 (145-2C11) (21), anti-CD9 (9D3) (11), anti-CD9 (KMC8) (22), anti-CD28 (Pv-1) (23), anti-CD5 (53-7-313) (24), anti-CD2 (RM2-2) (25), anti-CD44 (2B1-H2) (prepared in our laboratory), mouse anti-rat (MoR) (hybridoma cells MAR18.5) were obtained from American Type Culture

3 Abbreviations used in this paper: DIG, detergent-insoluble glycolipid-enriched; CTx, cholera toxin.
Preparation of a purified T cell population

Lymph node cells were depleted of B cells and Ig^+^ APCs by immuno-magnetic negative selection. Briefly, lymph node cells were allowed to react with anti-I-Ad/b Ab and then incubated with magnetic particles bound to goat anti-mouse Ig (Advanced Magnetics, Cambridge, MA). A T cell population depleted of anti-I-Ad/b-labeled and/or surface Ig^+^ cells was obtained by removing cells bound to magnetic particles with a rare earth magnet (Advanced Magnetics). Purity of the resulting population was checked by flow cytometry with anti-CD3. Purified T cells were consistently >98% CD3 positive.

Cell labeling

For surface biotinylation, purified lymph node T cells were washed three times in PBS and resuspended at the concentration of 1 x 10^7/ml in PBS and 0.2 µg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL). After a 30-min incubation at room temperature, the cells were washed three times with PBS to remove free biotin. Labeled cells were lysed with 1 ml of ice-cold MBS lysis buffer (MBS (25 mM MES, 150 mM NaCl, pH 6.5), 1% Triton X-100, 1 mM PMSF, 10 µg/ml aprotinin, and 1 µg/ml leupeptin).

Sucrose gradient centrifugation

To obtain the DIG/raft membrane fraction, purified lymph node T cells (2 x 10^8) were lysed with 1 ml of ice-cold MBS lysis buffer. Following a 30-min incubation on ice, the lysates were homogenized with 20 strokes of a loose-fitting Dounce homogenizer, gently mixed with an equal volume of 80% sucrose (w/v) in MBS, and placed in the bottom of a SW40Ti centrifuge tube. The sample was then overlaid with 6.5 ml of 30% sucrose and 3.5 ml of 5% sucrose in MBS (with 1 mM Na_3PO_4) and spun for 16—19 h at 100,000 x g at 4°C (in a Beckman SW40Ti) (Fullerton, CA). One-milliliter fractions were harvested serially from the top of the gradient. The DIG/raft fraction and cytosol/heavy membrane fraction were obtained in fractions 4—6 and 10—12, respectively.

Preparation of lysates from stimulated T cells

T cells were washed and resuspended at the concentration of 1 x 10^7/ml in RPMI 1640 medium containing 0.25 µg/ml or 5 µg/ml anti-CD3 mAb. After a 15-min incubation at 4°C, 10 µg/ml of Ab against various T cell molecules were added, and cells were incubated for an additional 15 min at 4°C. The cells were washed by RPMI 1640 and incubated with 20 µg/ml streptavidin for 30 min at 37°C. After stimulation, cells were pelleted and washed in buffer (ice-cold 0.2% BSA/PBS), and then incubated with 20 µg/ml RED670-conjugated streptavidin (Life Technologies) plus 5 µg/ml FITC-conjugated CTx at 37°C for 30 min to induce capping. After capping, cells were washed with ice-cold Hank's 0.1% sodium azide and then fixed immediately in 1 ml 4% paraformaldehyde in PBS to remove free biotin. Labeled cells were lysed with 1 ml of ice-cold MBS lysis buffer (MBS (25 mM MES, 150 mM NaCl, pH 6.5), 1% Triton X-100, 1 mM PMSF, 10 µg/ml aprotinin, and 1 µg/ml leupeptin).

Calcium mobilization assay

Resting lymph node T cells were suspended at 1 x 10^7/ml in 2% FCS/PBS containing 3 µM fura 2AM (Dojindo, Kumamoto, Japan) and incubated at 37°C for 30 min. Cells loaded with fura 2AM were then incubated with 10 nM methyl-β-cyclodextrin in 2% FCS/PBS for 30 min at 37°C. Fura 2-loaded cells were pelleted and washed twice and then resuspended at 5 x 10^6/ml in PBS containing 0.5 mM CaCl_2. The calcium response was initiated by biotinylated anti-CD3 mAb (0.25 or 5 µg/ml) and biotinylated anti-CD28 (20 µg/ml) against various T cell molecules plus 20 µM Ionomycin at 20°C temperature. In some experiments, immunofluorescence staining of intracellular molecules was performed as described (28). Briefly, fixed cells were permeabilized using 0.1% Triton X-100 in PBS, washed, and incubated with PBS/BSA for preblocking. Cells were then stained with first Ab followed by FITC-conjugated goat anti-rabbit Ab (Jackson Immunoresearch, West Grove, PA). Fixed cells were plated onto glass coverslips, mounted in glycerol/PBS, and examined on a Carl Zeiss microscope (Zeiss LSM410, Oberkochen, Germany) using an oil immersion lens. Appropriate excitation and barrier filters were used to observe fluorescence. Photographs of cells shown in figures represent the majority of cells displaying cell-surface staining patterns observed in these experiments.

Results

Various costimulatory molecules exist in the DIG fraction on the T cell

Recent studies have shown that the DIG fraction (also called raft), which is rich in T cell signaling molecules, has an important role in the generation of a TCR signal (18, 19). While TCR/CD3 complex itself does not exist in the DIG/raft fraction, TCR engagement results in the recruitment of TCR/CD3 to DIG/rafts (18, 19). In view of the role for DIG/rafts in TCR signaling, we first examined whether various costimulatory molecules exist inside and/or outside the DIG/raft fraction. Purified lymph node T cells were lysed in a buffer containing nionic detergent, and DIG/rafts were fractionated by sucrose gradient centrifugation. Consistent with the previous studies (29, 30), the DIG fractions were enriched for glycosphingolipids as determined by reactivity with CTx, which recognizes ganglioside GM1 (Fig. 1A). Several TCR-signaling molecules have also been shown to localize specifically in the DIG fraction of T cells (31—33). For example, the localization of Lck in the DIG fraction is confirmed in Fig. 1A. Moreover, Fig. 1A shows that CD5, CD9, and CD44, which have been known as costimulatory molecules, exist in the DIG fraction, although they are also present in the cytosol and heavy membrane fraction (thereafter described as the heavy membrane fraction). The observation that...
CD44 is present in the DIG/raft fraction is consistent with a previous report (34). Although a large portion of CD5 existed in the non-DIG fraction as previously described (35), our results indicate that a significant albeit small portion of CD5 is present in the DIG/raft fraction.

Because anti-CD28 (Pv-1) was not usable for Western blotting, the existence of CD28 in the DIG fraction was examined by cell-surface biotinylation and immunoprecipitation. This was done in parallel to the detection of CD2 and CD5 as a positive control of DIG-resident molecules by immunoprecipitation using the corresponding mAb. T cells were surface biotinylated and lysed in detergent-containing buffer. Each fraction was precipitated with the indicated Ab-bound beads, and bound proteins were subjected to SDS-PAGE. CD5 was again detected in the DIG fraction by surface biotinylation and immunoprecipitation. CD2 was also detected by this procedure. Although a previous paper (35) described the partition of CD2 mainly in the non-DIG fraction, our data showed that a large portion of CD2 exists in the DIG fraction. In contrast, the amount of CD28 detected in the DIG fraction was only marginal, and the vast majority of this molecule was found in the heavy membrane fraction.

Ligation of DIG-resident costimulatory molecules induces the redistribution of DIG

Various GPI-linked proteins such as Thy-1 and CD48 are present preferentially in the DIG fraction (36). Previous reports illustrated that the ligation of Thy-1 or CD48 molecules using mAb induces

![Figure 1](image1.png)

**FIGURE 1.** The presence of various non-CD28 costimulatory molecules in the DIG/raft fraction. A, The lysate from resting T cells was fractionated on a sucrose density gradient as described in Materials and Methods. Protein contents of each fraction of the sucrose gradient were resolved on SDS-PAGE (an equal volume of material from the different fractions were loaded), and protein patterns were analyzed on immunoblots stained with the corresponding Abs or HRP-CTx reactive with GM1 as indicated. The label “heavy” indicates the heavy membrane fraction. B, Resting T cells were surface-biotinylated. The lysate prepared from biotinylated cells was fractionated on a sucrose density gradient. Each fraction was precipitated with the indicated Ab-bound beads, and bound proteins were subjected to SDS-PAGE. C, T cells were stimulated with 0.25 μg/ml biotinylated anti-CD3 alone or with 5 μg/ml biotinylated anti-CD5 mAb followed by streptavidin (20 μg/ml) for 30 min at 37°C. The lysates from these stimulated cells were fractionated. Data are representative of four (A), three (B), and two (C) similar experiments.

![Figure 2](image2.png)

**FIGURE 2.** Collection of DIG/rafts to the site of engagement of DIG/raft-resident costimulatory molecules. T cells were stained with GM1-reactive FITC-CTx alone (upper left panel) or simultaneously with FITC-CTx (right in each set of panels) and mAb against indicated costimulatory molecules followed by cross-linking with RED670-streptavidin (left in each set of panels) for 30 min at 37°C. Stained cells were examined on a confocal microscopy. Data are representative of five similar experiments.
the redistribution of DIG/rafts along with the aggregation of Thy-1 (37) or CD48, respectively (38). We examined whether DIG/raft-resident costimulatory molecules, when ligated, can also affect the redistribution of DIG/rafts. For a raft marker, we used GM1-reactive FITC-CTx. T cells were simultaneously incubated with combinations of FITC-CTx and either of various biotinylated mAbs against DIG/raft-resident costimulatory molecules (anti-CD5, anti-CD9, anti-CD2, and anti-CD44) or of FITC-CTx and anti-CD28 followed by further cross-linking with RED670-conjugated streptavidin. Treatment of T cells with FITC-CTx alone (Fig. 2, upper left) or together with each mAb (data not shown) resulted in a diffuse or ring pattern of plasma membrane staining. Incubating the mAb-stained cells at 37°C for 30 min in the presence of streptavidin led to the collection of the relevant cell-surface molecules (CD5, CD9, CD2, and CD44) to polarized surface caps (Fig. 2). GM1 stained with FITC-CTx also produced a cap, and extensive overlaps of GM1 and each of the above molecules were observed (Fig. 2). For example, following the ligation of CD5, >50% of cells exhibited the overlap. These results indicate that the ligation of DIG/raft-resident costimulatory molecules induces the redistribution of DIG/rafts along with their own mobilization. However, Fig. 2 also shows that the ligation of CD28 not present in the DIG/raft induced moderate levels of DIG/raft aggregation along with the collection of CD28, although the incidence of cells exhibiting the overlap was lower than that of the overlap in anti-CD5-ligated cells and ∼30%.

FIGURE 3. CD5 costimulation induces enhanced Ca\(^{2+}\) influx by T cells stimulated with a suboptimal dose of anti-CD3. Intragelular free calcium levels in fura 2-loaded cells were monitored by a spectrophotometer after stimulation with 10 μg/ml biotinylated mAbs (indicated) together with 0.25 μg/ml biotinylated anti-CD3 or with 5 μg/ml biotinylated anti-CD3 alone followed by streptavidin (20 μg/ml). The first and second arrows in each panel indicate the time point for the addition of biotinylated mAbs and streptavidin, respectively. Data are representative of three similar experiments.

FIGURE 4. Coligation of CD3 and CD5 enhances the association of CD3 and DIG/rafts. T cells were incubated with biotinylated anti-CD3, biotinylated anti-CD5, or biotinylated anti-CD28 alone or with a combination of anti-CD3 and anti-CD5 or anti-CD28 followed by cross-linking with RED670-streptavidin. A, Panels b and d show the aggregation of CD5 and CD3/CD5, respectively, stained with RED670. CD3 accumulation was analyzed by FITC-conjugated anti-hamster IgG (a, c, and e). B, RED670 staining shows the aggregation of CD3 (a), CD5 (c), CD3/CD5 (e), CD28 (g), or CD3/CD28 (i). DIG/raft (GM1) accumulation was analyzed by FITC-CTx staining (b, d, f, h, and j). C, The percentages of cells exhibiting the overlap of GM1 and CD3, CD5, CD28, CD3/CD5, or CD3/CD28 (among 50 cells examined in each group) in the protocol of B are shown. Data are representative of five similar experiments. Data from two independent experiments are presented in C.
Enhanced association of CD3 and DIG/rafts by coligating CD3 and DIG/raft-resident costimulatory molecules

We examined whether coligation of CD3 and DIG/raft-resident costimulatory molecules is capable of enhancing the recruitment of CD3 to DIG/rafts. In the following experiments, we chose CD5 as a model of DIG/raft-resident costimulatory molecules. Our previous results showed that costimulation of resting T cells with anti-CD5 plus suboptimal doses of anti-CD3 results in striking enhancement of T cell activation compared with that induced with anti-CD3 alone (12). Fig. 3 shows that coligation with anti-CD5 and a suboptimal dose (0.25 μg/ml) of anti-CD3 induced high levels of Ca\(^{2+}\) mobilization under conditions in which stimulation with anti-CD3 alone was effective to a lesser or marginal extent, respectively, which is in accord with a previous report (39). A high dose (5 μg/ml) of anti-CD3 induced high levels of Ca\(^{2+}\) mobilization under conditions in which stimulation with anti-CD3 or anti-CD5 mAb alone induced mobilization Ca\(^{2+}\) to a lesser extent, respectively, which was accordant with the previous report (39). A high dose (5 μg/ml) of anti-CD3 induced potent Ca\(^{2+}\) influx without requiring costimulation. Therefore, the conditions using a suboptimal dose of anti-CD3 were used to determine the effect of the CD3-CD5 coligation on the redistribution of DIG/rafts. As shown in Fig. 4A, ligation with anti-CD3 (a suboptimal dose) or anti-CD5 (an adequate amount) alone led to the formation of a sparse (Fig. 4A, a) or dense cap (Fig. 4A, b), respectively, and coligation of CD3 and CD5 produced a more condensed cap (Fig. 4A, d). CD3 cap accumulated weakly in association with CD5 engagement by anti-CD5 alone (Fig. 4A, c), whereas an apparently enhanced amount of CD3 accumulated following engagement by anti-CD3 and anti-CD5 (Fig. 4A, e), indicating that coligation forms a CD3-CD5 cocap. The CD3 cap produced by ligation with anti-CD3 alone was marginally associated with GM1 capping (Fig. 4B, b), whereas anti-CD5 ligation again induced potent collection of GM1 (Fig. 4B, d). Through such an effect, coligation of CD3 and CD5 resulted in more potent recruitment of GM1 to the cocap of CD3/CD5 (Fig. 4B, f). These observations indicate that ligation of CD3 to CD5 induces enhanced association of CD3 and DIG/rafts. Similar effects were observed for the ligation of CD3 and other non-CD28 costimulatory molecules (CD9, CD2, and CD44) (data not shown). It should be noted that coligation of CD3 and CD28 also enhanced the association of CD3 and DIG/rafts (Fig. 4B, g and j), although CD28 still remained in the heavy membrane fraction (Fig. 1C), which is consistent with the observation of Viola et al. (20). The percentages of cells exhibiting the overlap of GM1 and CD3/CD5 or CD3/CD28 are summarized in Fig. 3C.

CD3/CD5 coligation induces accumulation of TCR-associated signaling molecules and its activation along with raft redistribution

The linker molecule LAT that exists in the DIG/raft fraction is a critical substrate of the tyrosine kinase ZAP-70 activated upon TCR engagement (40). We examined whether CD3/CD5 coligation induces the association of these TCR signaling molecules with the CD3/CD5 cocap that colocalizes with DIG/rafts. As shown in Fig. 5, the accumulation of ZAP-70 and LAT was observed in association with the CD3/CD5 cocap in the anti-CD3/anti-CD5 coligated T cell.
We next determined whether the above TCR signaling molecules associated with the CD3/CD5 cocap are activated for signal transduction. As shown in Fig. 6A, it was found using unfractionated T cell lysates that stimulation with a suboptimal dose of anti-CD3 alone induced only marginal levels of LAT phosphorylation and that coligation of CD3 and CD5 or CD9 strikingly enhanced the levels of LAT phosphorylation, while costimulation with anti-CD28 moderately up-regulated LAT phosphorylation. The lysates of T cells unstimulated or stimulated with anti-CD3 or anti-CD3/anti-CD5 were fractionated, and after confirming the previous results (41) that LAT is largely in the DIG fraction whereas ZAP-70 exists exclusively in the heavy membrane fraction (data not shown), the phosphorylation of LAT and ZAP-70 was examined separately in the DIG and heavy membrane fractions, respectively. Fig. 6B shows that coligation of CD3 and CD5 induces the phosphorylation of LAT in the DIG/raft fraction as well as of ZAP-70 in the heavy membrane fraction. These results support the notion that the DIG/raft-resident costimulatory molecule CD5 functions to strengthen the generation of a TCR signal through enhanced association of TCR/CD3 and DIG/rafts.

Disrupting the structure of DIG/rafts impairs Ca$^{2+}$ mobilization induced by coligation of CD3 and CD5

To determine whether the recruitment of DIG/rafts to the CD3/CD5 cocap is a prerequisite for T cell activation based on CD5 costimulation, we employed an approach to perturb DIG function before TCR stimulus. Methyl-β-cyclodextrin is known to disrupt the structure of DIG/rafts by extracting cholesterol from the plasma membrane (42, 43). T cells were treated with this reagent and, after washing, coligated with anti-CD3 plus anti-CD5 (Fig. 7). Without methyl-β-cyclodextrin treatment, coligation again induced the recruitment of GM1 to the cocap of CD3-CD5 (Fig. 7, a and b). The same protocol of coligation produced the cocap of CD3 and CD5 in methyl-β-cyclodextrin-pretreated T cells, whereas the redistribution of DIG/rafts was not induced (Fig. 7, c and d). Thus, the association of CD3 and GM1 no longer follows coligation of CD3 and CD5 in the absence of intact rafts. Similar observations were made for the ligation of CD3 and CD28 (Fig. 7, e–h).

Fig. 8 shows the effect of DIG disruption on Ca$^{2+}$ mobilization induced in resting T cells by CD5 costimulation. Under conditions in which stimulation with a suboptimal dose of anti-CD3 alone induced low levels of Ca$^{2+}$ influx, CD5 costimulation again elicited enhanced Ca$^{2+}$ mobilization. Such an enhancement was also induced by CD28 costimulation. Importantly, enhanced Ca$^{2+}$ responses in both costimulation were prevented by pretreatment with methyl-β-cyclodextrin. Taken together, the results suggest that Ca$^{2+}$ mobilization enhanced by CD5 costimulation is not based simply on the aggregation of CD3 and CD5 molecules but essentially

**FIGURE 7.** Effects of treatment with a DIG/raft-disrupting agent on the cocapping of CD3/CD5 and the redistribution of DIG/rafts. T cells were untreated or treated with 10 mM methyl-β-cyclodextrin for 30 min at 37°C and then coligated with anti-CD3 plus anti-CD5 or anti-CD28. Data are representative of three similar experiments.

**FIGURE 8.** Effect of treatment with a DIG/raft-disrupting agent on Ca$^{2+}$ influx. T cells were untreated or treated with 10 mM methyl-β-cyclodextrin for 30 min at 37°C. Cells were stimulated with anti-CD3 alone or together with indicated mAb or with ionomycin (50 ng/ml) as a control stimulator to confirm cell viability and activity. Data are representative of three similar experiments.
Differential induction of Ca$^{2+}$ influx and LAT phosphorylation by coligation or independent ligation of CD3 and CD5. T cells were stimulated with biotinylated anti-CD3 and biotinylated anti-CD5 followed by cross-linking with streptavidin (coligation). For independent ligation, either CD3 or CD5 was ligated with biotinylated Ab/streptavidin or two sets of Abs (anti-CD3 plus goat anti-hamster (GoH) Ab or anti-CD5 plus mouse anti-rat (MoR) Ab). These stimulated T cells were subjected to Ca$^{2+}$ mobilization assays (A) and the determination of LAT phosphorylation (B). Data are representative of three similar experiments.

**FIGURE 9.** Differential induction of Ca$^{2+}$ influx and LAT phosphorylation by coligation or independent ligation of CD3 and CD5. T cells were stimulated with biotinylated anti-CD3 and biotinylated anti-CD5 followed by cross-linking with streptavidin (coligation). For independent ligation, either CD3 or CD5 was ligated with biotinylated Ab/streptavidin or two sets of Abs (anti-CD3 plus goat anti-hamster (GoH) Ab or anti-CD5 plus mouse anti-rat (MoR) Ab). These stimulated T cells were subjected to Ca$^{2+}$ mobilization assays (A) and the determination of LAT phosphorylation (B). Data are representative of three similar experiments.

**Discussion**

The fate of TCR-stimulated T cells depends largely on whether their costimulatory receptor(s) is simultaneously stimulated to produce costimulatory signal(s) responsible for inducing T cell activation (1–4). While CD28 has been recognized as the principal costimulatory receptor for T cell activation (2–4), a number of other molecules have also been described to possess costimulatory capacity, including CD5 (8), CD2 (9), CD9 (11), CD4 (10), CD43 (44), and CD11a (45). Although their capacity to costimulate T cells has been well established, the mechanism of costimulation is less understood.

In this report, we have shown that a number of the thus far described non-CD28 costimulatory molecules exist in the DIG/raft fraction of T cell plasma membranes and that the ligation of such DIG/raft-resident costimulatory molecules (for example, CD5) induces the mobilization of DIG/rafts. More importantly, coligation of CD3 and CD5 led to increased Ca$^{2+}$ mobilization compared with ligation of CD3 alone, and such a coligation induced the enhanced association of CD3/TCR and DIG/rafts based on the redistribution of DIG/rafts accompanying CD5 aggregation. CD28 exists in the DIG/raft fraction of the T cell in a negligible amount. However, consistent with a recent paper (20), the engagement of this molecule resulted in comparable levels of DIG/raft-redistribution to those induced by CD5 engagement. This suggests that the mechanism underlying CD28-mediated DIG-redistribution may be distinct from that for the aggregation of DIG/rafts induced using Ab such as anti-CD5. Treatment of T cells with an agent capable of disrupting DIG structure/function before the ligation of CD3 and CD5 resulted in the impaired recruitment of CD3 to DIG/rafts as well as complete inhibition of Ca$^{2+}$ mobilization. These observations are in accord with the recent proposition that the recruitment of TCR/CD3 to DIG/rafts represents an important biochemical event in TCR-mediated signal transduction (18, 19), and provide a mechanism for T cell costimulation mediated by costimulatory molecules present in the DIG fraction.

The plasma membrane bilayer contains fractions with distinct compositions and functions (46, 47). Among them, the detergent-insoluble glycolipid-enriched (DIG) fraction, also called as the sphingolipid-cholesterol raft, has been proposed to be involved in various cell events including signal transduction (36, 48). A variety of signaling molecules are concentrated in the DIG/raft fraction, including src family kinases such as Lck and molecules involved in Ca$^{2+}$ influx (19, 48). The aforementioned two studies (18, 19) made essentially the same observation that TCR engagement promotes a selective association of TCR/CD3 complexes with DIG/rafts. The recruitment of TCR/CD3 to DIG/rafts occurred along with the high enrichment of phosphorylated ζ p23 and the concentration of activated ZAP-70 complex with TCR/CD3. Among various protein tyrosine kinases present in the T cell, Lck is the key protein tyrosine kinase that activates the TCR/CD3/ZAP-70 complex in TCR-dependent signaling pathways (49). Thus, early TCR-signaling events are achieved when TCR/CD3 and DIG/raft-associated Lck are brought into close proximity as a result of TCR recruitment to DIG/rafts. It is also possible that recruitment of TCR/CD3 is the consequence of TCR/CD3 oligomerization (50, 51) because such an oligomerization could strengthen the association of TCR/CD3 with DIG/rafts (36).

It is obvious that the stimulation of T cells with high concentrations (5 or 10 μg/ml) of anti-CD3, even alone, is sufficient to induce high levels of LAT phosphorylation was seen only by coligation of CD3 and CD5 (Fig. 9B).
induce the association of TCR/CD3 with DIG rafts as well as pot-ent Ca\(^{2+}\) influx. However, stimulation of T cells with a small amount of anti-CD3 alone induced only weak levels of colocal-ization of TCR/CD3 and DIG rafts. High levels of colocalization were achieved when such an anti-CD3 stimulation was combined with costimulation of CD5 as a DIG/rafts-resident molecule. This enhanced colocalization led to the up-regulation of Ca\(^{2+}\) influx through the activation of TCR-associated signaling molecules such as ZAP-70 and LAT. LAT is present in the DIG fraction (41), whereas a large portions of ZAP-70 exists in the non-DIG fraction (41). In fact, phosphorylated LAT and ZAP-70 were recovered from the DIG and non-DIG fractions, respectively, of the lysates of anti-CD3/anti-CD5-stimulated T cells. However, it should be noted that, like LAT, ZAP-70 accumulated in association with DIG rafts redistributing after CD3/CD5 coligation. When the as-sociation of TCR/CD3 with DIG rafts was inhibited by disrupting the structure of DIG rafts, CD5 costimulation failed to enhance Ca\(^{2+}\) mobilization. Importantly, the capping of CD3 and CD5 was not affected under these conditions. Thus, the colocalization of CD5 molecules themselves with TCR is insufficient to generate a TCR signal leading to Ca\(^{2+}\) mobilization. Instead, our present re-sults suggest that the costimulatory function of DIG/raft-resident costimulatory molecules is to promote the association of TCR/ CD3 with DIG rafts and to induce enhanced tyrosine phosphory-lation of TCR-associated signaling molecules. These observations are consistent with the suggestion of Moran and Miceli (38) that raft-associated accessory molecules function to augment TCR sig-naling through lipid raft redistribution and activation of protein tyrosine kinases.

It would be of importance to compare the mechanism of co-stimulation induced by DIG/raft-resident costimulatory molecules and the principal costimulatory molecule CD28. Viola et al. showed that CD28 engagement induces the redistribution of DIG/ rafts (20). Although they did not examine the existence of CD28 in rafts, their study suggested that in contrast to the clustering of rafts by ligation of raft-associated molecules, CD28 reorganizes rafts into an active configuration (20). Our present results illustrate that the amount of CD28 present in the raft is quite low or negligible compared with that of CD5. Nevertheless, CD28 engagement induces comparable levels of raft redistribution and Ca\(^{2+}\) influx to those induced by CD5 ligation. Together, these results suggest that the ligation of DIG/raft-resident molecules induces the passive (Ab-ligated) redistribution of rafts, whereas CD28 engagement leads to the reorganization of rafts through a different mechanism.

The latter may represent the enhancement of the mechanism that underlies the association of TCR/CD3 and rafts by ligation with a high dose of anti-CD3 (18, 19). This might also be related to the widely accepted observation that CD28 molecules function to co-stimulate T cells without being cross-linked, whereas non-CD28 molecules require their cross-linking for costimulation.

Finally, the role of raft redistribution should be considered in the light of overall requirements in the full T cell activation process. Our previous studies demonstrated that CD28- and non-CD28 molecule-costimulated T cells exhibited almost the same levels of \([\text{H}]\text{Thymidine incorporation, whereas they greatly differed in IL-2 production and cellular proliferation}\) (12). This indicated that there exists a fundamental difference in the mechanism of T cell co-stimulation between CD28 and non-CD28 molecules. Full T cell activation is achieved by a combination of a TCR-derived signal and a costimulatory signal distinct from a TCR signal. The present study shows that the clustering of DIG rafts based on cross-linking of DIG/raft-resident costimulatory molecules as well as of CD28 molecules existing largely outside DIG rafts contributes to en-hanced generation of a TCR signal as measured by Ca\(^{2+}\) influx.


