# VLA-4 Mediates CD3-dependent CD4+ T Cell Activation Via the CS1 Alternatively Spliced Domain of Fibronectin

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## Summary

We previously showed that fibronectin (FN) synergized with anti-CD3 in induction of CD4+ T cell proliferation, and that VLA-5 acted as a functional FN receptor in a serum-free culture system. In the present study, we showed that VLA-4 is also involved in this CD3-dependent CD4 cell activation through its interaction with the alternatively spliced CS1 domain of FN. When highly purified CD4 cells were cultured on plates coated with anti-CD3 plus synthetic CS1 peptide-IgG conjugate, significant proliferation could be observed. Neither CS1 alone nor anti-CD3 alone induced this activation. This proliferation was completely blocked by anti-VLA$\beta$1 (4B4) and anti-VLA-4 (8F2), while anti-VLA-5 (monoclonal antibody [mAb] 16 and 2H6) had no effect. These data indicate that VLA-4 mediates CD3-dependent CD4 cell proliferation via the CS1 domain of FN. Anti-VLA-4 also partially (10–40%) inhibited CD4 cell proliferation induced by native FN plus anti-CD3, implying that the CS1 domain is active in the native plasma FN. However, this native FN-dependent proliferation was entirely abolished by addition of anti-VLA-5 alone. Moreover, when native FN-coated plates were pretreated with anti-FN (mAb 333), which blocks RGDS sites but not CS1 sites, no CD4 cell activation could be observed. These results strongly suggest that CD4 cell activation induced by plasma FN/anti-CD3 may be dependent on both VLA4/CS1 and VLA5/RGDS interactions, although the latter interaction may be required for function of the former.

It is now evident that CD4+ T cells can be divided into two distinct functional subsets based upon their expression of different isoforms of the leukocyte common antigens/T200 family (CD45/CD45RA) (1–3) and the CD29 (4B4) antigen (4). CD4+CD45RA−CD29+(CD29$^{high}$) cells provide strong helper function for B cell Ig production, respond maximally to recall antigens (4), and induce CD8 cells to exert class I–restricted cytotoxicity (5). The CD4+ CD45RA+CD29−(CD29$^{low}$) cells, in contrast, can induce CD8 cells to suppress Ig synthesis but provide poor helper function, and respond poorly to recall antigens. The CD29 (4B4) antigen has been shown to belong to the VLA/integrin super gene family (6). These transmembrane glycoproteins are expressed on a variety of cell types including lymphocytes. The VLA protein family consists of a common $\beta$1 subunit noncovalently associated with different $\alpha$ chains to form different heterodimers (7, 8). At least six VLA proteins have been identified based on their distinct $\alpha$ chains. Most of these VLA proteins are known to function as cell surface receptors for extracellular matrix proteins, including fibronectin, collagen, and laminin. Thus, they are considered to play a significant role in a variety of biological processes, including cell migration, embryogenesis, and tumor metastasis (7, 8). However, their function in the immune system is largely unknown. While CD29 expression correlates with the function of subsets of CD4 cells, the mechanism underlying this relationship is still unclear.

Our recent observation (9) that fibronectin (FN)[1] synergizes with the CD3/TCR pathway to promote CD4+ T

[1] *Abbreviations used in this paper:* FN, fibronectin; SPDP, *N*-succinimidyl-3(2-pyridylithio) propionate.

cell proliferation may provide important insights into the mechanism by which extracellular matrix proteins modulate immune function. When plasma FN is crosslinked with anti-CD3 on culture plates, highly purified CD4 cells were maximally activated in a serum-free culture system. This anti-CD3/FN-dependent proliferation was specifically inhibited by anti-4B4 (anti-VLA-$\beta$1) and anti-VLA-5 antibodies, suggesting that VLA-5 was acting as a major functional FN receptor in our system. It has subsequently been demonstrated that VLA-4 also functions as another type of FN receptor (10–12). While the binding of VLA-5 is mediated by an Arg-Gly-Asp-Ser (RGDS)-containing cell binding domain in the central portion of FN (13, 14), that of VLA-4 to FN is mediated by an alternatively spliced domain (CS1) of the FN molecule (10). Since CS1 is different from RGDS in its sequence (10, 15, 16), the FN molecule has at least two distinct cell binding domains. In contrast to other VLA proteins, which are distributed widely on a variety of cell types, the expression of VLA-4 is relatively restricted to myeloid and lymphoid cells (7). It is therefore conceivable that VLA-4 might have a significant role in T cell function through its interaction with FN. We now present evidence that immobilized synthetic CS1 peptides, via their interaction with the VLA-4 receptor, provide a signal synergistic with anti-CD3 in promoting CD4 cell proliferation. Moreover, we demonstrate that CD4 cell proliferation induced by anti-CD3 plus native plasma FN is dependent on both VLA-4 and VLA-5, although the latter may be required for full function of the former.

## Materials and Methods

*Reagents.* Human FN was obtained from Collaborative Research, Lexington, MA. BSA, transferrin, and soybean lipids used in the serum-free medium were from Boehringer Mannheim Biochemicals, Mannheim, FRG. IMDM was from Sigma Chemical Co., St. Louis, MO.

*Antibodies.* mAbs reactive with the lymphocyte surface antigens, CD3 (RW24B6, IgG2b; OKT3, IgG2a), CD8 (21Thy-2D3, IgG1), CD11a (2F12, IgG1), CD11b (Mol, IgM), CD29 (4B4, IgG1), have been described previously (4, 17–19). Anti-8F2 is reactive with the $\alpha$4 subunit of VLA (anti-VLA-4), and has been described previously (9). mAb 16 is an IgG2a of rat origin and recognizes the $\alpha$5 VLA subunit (anti-VLA-5) (9). Anti-2H6 antibody (IgG1) was developed in our laboratory by standard hybridoma techniques after immunization of a mouse with the K562 cell line. It was determined that anti-2H6 reacted with the VLA-5 protein as follows. The protein immunoprecipitated by anti-2H6 had the same molecular weight as that immunoprecipitated by the mAb 16 antibody. Moreover, sequential immunoprecipitation studies showed that both antibodies reacted with the same molecule (data not shown). mAb 333 is a rat anti–human FN antibody and was characterized previously (20). This antibody binds close to the RGDS sequence of the FN molecule and blocks its function.

*Preparation of CS1 Peptides.* The alternatively spliced CS1 domain of the type III connecting segment (IIICS) of human FN, as well as truncated and scrambled homologues, were prepared as described previously (15, 16). Fig. 1 shows the amino acid sequences of the CS1 peptide and related peptides used in our study. According to an adhesion assay using melanoma cell lines, the LDV tripeptide is the most important region for the promotion of adhesion by CS1 (Komoriya et al., manuscript in preparation). CS1-D and CS1-A, as well as the original CS1 peptide, contain this sequence, and therefore exhibit cell attachment capabilities. Their relative activity, as determined by the melanoma cell adhesion assay, is CS1 > CS1-D > CS1-A. In contrast, CS1-B and CS1-scrl do not contain the LDV tripeptide, and therefore have no adhesive activity. They are used as negative controls in our study. It should be noted that CS1-scrl is identical to CS1 except in its final eight amino acids (Fig. 1, underlined), which are scrambled such that no amino acid retains its original position. For T cell adhesion and proliferation assays, CS1 peptides were covalently coupled to rabbit IgG using heterobifunctional crosslinker *N*-succinimidyl-3(2-pyridyldithio) propionate (SPDP), as described elsewhere (15, 16) In brief, SPDP was dissolved in ethanol at a concentration of 1.5 mg/ml and mixed with 6 mg/ml rabbit IgG (Miles Scientific, Naperville, IL) in Dulbecco's PBS to give an SPDP/IgG weight ratio of 1:10 (molar ratio of 50:1). After 30 min of incubation at room temperature, unreacted SPDP was removed by gel filtration on a PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS. The activated IgG was then added to CS1 peptide at a peptide/IgG weight ratio of 0.3–0.45. After mixing overnight on a rotator at room temperature, free peptide was removed by dialysis against PBS. To determine the amount of CS1 in the conjugate, [$^3$H]CS1 peptides were used as a carrier, as described previously (16). The number of peptide molecules/IgG molecule was found to be ~15 for each CS1-IgG conjugate.

*Preparation of Cells.* Human PBL were isolated from healthy donors as described previously (9). Briefly, the lymphocytes were separated into erythrocyte rosette–positive (T cells) and –negative populations with sheep erythrocytes. The T cells were depleted of contaminating monocytes by adherence to plastic plates. Further removal of monocytes from T cells was achieved by incubation with 5 mM L-leucine methyl ester HCl. CD4$^+$ T cells were obtained by incubating T cells with anti-CD8, anti-CD11b, and L243, and panning on anti-Ig-coated petri dishes to remove residual monocytes. The CD4 cells thus obtained were >90% CD4$^+$, with <5% contamination by CD8$^+$ cells. The contamination by monocytes was <1 in 500 cells as determined by peroxidase staining.

*Cell Culture and Proliferation Assays.* For preparation of microtiter plates coated with anti-CD3 plus CS1-IgG or anti-CD3 plus FN, 100 $\mu$l of protein A–purified anti-CD3 antibody at 0.1 $\mu$g/ml in PBS was plated in each well of a 96-well flat-bottomed plate (3595; Costar, Cambridge, MA), which was then incubated for 3 h at room temperature. After washing twice with PBS, 100 $\mu$l of PBS containing the indicated amounts of CS1-IgG or FN were then plated in each well and incubated for 3 h at room temperature. Before use, wells were washed three times with PBS. T cells were cultured in triplicate wells at a concentration of $10^5$ cells/well in serum-free medium consisting of Iscove's MEM supplemented with 0.1% BSA, 30 $\mu$g/ml human transferrin, 10 $\mu$g/ml soybean lipids, 50 $\mu$g/ml cholesterol, 4 mM L-glutamine, 25 mM Hepes buffer (Microbiological Associates, Bethesda, MD), 0.5% sodium

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|---|---|
| CS1 | CDELPQLVTLPHPNLHGPEILDVPST |
| CS1-D | CVTLPHPNLHGPEILDVPST |
| CS1-A | EILDVPST |
| CS1-B | CDELPQLVTL |
| CS1-scrl | CDELPQLVTLPHPNLHGPPVTSELID |

**Figure 1.** Amino acid sequence of CS1 and its related peptides. CS1 contains residues 1–25 of the type III connecting segment of FN, and comprises an entire spliced segment. CS1-scrl was designed to have the same sequence as CS1, except for the underlined segment in which eight amino acids were scrambled such that no amino acid retained its original position.

bicarbonate, and 50 μg/ml of gentamicin sulfate (Schering, Kenilworth, NJ). After 4 d in culture, each well was labeled with 1 μCi of [$^3$H]thymidine (ICN Radiochemicals, Irvine, CA) for 18 h. Cells were then harvested on a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA), and [$^3$H]thymidine incorporation was measured with a β scintillation counter. For antibody inhibition assays, cells were preincubated with media containing various amounts of antibodies for 30 min at room temperature before being added to the ligand-coated wells in the continued presence of the antibodies.

*Cell Adhesion Assay.* Cell adhesion assays were performed according to the method of Yamada and Kennedy (21) with minor modifications. In brief, 96-well microtiter plates were incubated with 100 μl of CS1-IgG conjugate for 3 h at room temperature, followed by incubation with heat-treated BSA (10 min at 80°C) in PBS (5 mg/ml) for 2 h at room temperature and washing with PBS. Jurkat cells were labeled with [$^3$H]thymidine for 6 h before the adhesion assay, and were then plated on CS1-IgG-coated plates, and incubated for 1 h at 37°C. The unbound cells were removed by washing with PBS. The bound cells were lysed with 2% SDS and 0.1% NaOH, and the radioactivity in the cell lysates was analyzed using a β scintillation counter.

## Results

*CS1 Peptides Synergize with Anti-CD3 to Promote CD4$^+$ T Cell Proliferation.* Our previous study (9) demonstrated that CD4 cells were activated when they were cultured on plates coated with both anti-CD3 and FN in a serum-free culture medium. Moreover, VLA-5 was shown to operate as a functional FN receptor in this system. However, recent evidence has indicated that VLA-4 acts as a lymphocyte receptor for the alternatively spliced CS1 domain of plasma FN (10, 11). To determine whether this CS1 domain of plasma FN can also provide a complementary signal with anti-CD3 in the activation of CD4 cells, we studied the effect of the synthetic CS1 peptide on the proliferation of highly purified CD4 cells in a serum-free culture system.

As shown in Fig. 2, no proliferation above background was observed when CD4 cells were cultured with anti-CD3 antibody alone or CS1 peptide conjugate alone. In contrast, when CD4 cells were cultured on plates coated with both anti-CD3 and CS1, marked proliferation was observed. The control peptide, CS1-scrl, had no effect, even when combined

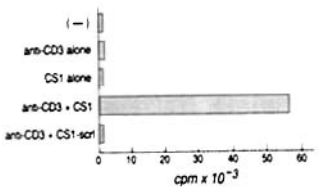


**Figure 2.** Comitogenic effect of CS1 peptide on CD3-dependent CD4 cell proliferation. CD4 cells were cultured on plates coated with anti-CD3 (0.1 μg/ml), CS1 (2.5 nmol/ml), or CS1-scrl (2.5 nmol/ml), alone or in combination. After 4 d of culture, proliferation was assessed by determining [$^3$H]thymidine incorporation. The data are expressed as the mean of triplicate samples. Each SD was <15%. The data shown are representative of five separate experiments.

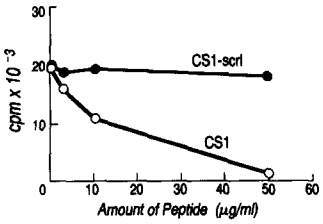


**Figure 3.** Soluble CS1 peptide inhibits the proliferation induced by anti-CD3 plus immobilized CS1 peptide. CD4 T cells were incubated with various concentrations of soluble CS1 or CS1-scrl peptides for 30 min, and then placed onto culture plates coated with anti-CD3 (0.1 μg/ml) and CS1 peptide (0.5 nmol/ml). After 4 d, proliferation was assessed by determination of [$^3$H]thymidine incorporation. The data are expressed as the mean of triplicate samples. Each SD was <15%. The data shown are representative of three separate experiments.

with anti-CD3. These results indicate that the combination of CS1 peptide and anti-CD3 is a potent activator for CD4 cells. As shown in Fig. 3, the addition of soluble CS1 peptide to the culture medium resulted in inhibition of CD4 cell proliferation induced by anti-CD3 plus CS1, in a dose-dependent fashion. The control peptide, CS1-scrl, did not have any effect. These results suggest that the interaction of CS1 and its receptor may be important in the CD4 cell activation observed.

*Anti-4B4 (Anti-VLAβ1) and Anti-8F2 (Anti-VLAα4) Inhibit Anti-CD3/CS1-Dependent CD4 Cell Proliferation.* Cell adhesion assays (10–12, 15, 16) have recently provided convincing evidence that the CS1 domain of the FN molecule recognizes the VLA-4 protein. Therefore, we next examined whether the proliferative response of CD4 cells in our system was also mediated by a CS1/VLA-4 interaction, by performing an antibody inhibition study. As shown in Fig. 4, anti-VLA-β1 (anti-4B4) and anti-VLA-4 (anti-8F2) antibodies almost completely inhibited the proliferation of CD4 cells activated by anti-CD3 plus CS1. In contrast, neither anti-VLA-5 (anti-2H6 or mAb 16) antibodies nor anti-LFA1 (anti-2F12) antibody (isotype control, IgG1) had any effect at concentrations up to 10 μg/ml. Likewise, antibodies against other VLA family antigens (VLA 1, 2, 3, and 6) did not inhibit anti-CD3 plus CS1-mediated CD4 cell proliferation (data not shown). On the other hand, anti-4B4 and anti-8F2 antibodies failed to inhibit T cell proliferation induced by the combination of anti-T11$_2$ and anti-T11$_3$ (data not shown). These results suggested that the interaction of CS1 and VLA-4 is specifically involved in CD4 cell activation induced by anti-CD3 plus CS1.

*CS1 Promotes Jurkat T Cell Adhesion, Which Is Specifically Blocked by Anti-4B4 and Anti-8F2 Antibodies.* The above study implied that anti-4B4 and anti-8F2 might inhibit T cell activation by blocking the binding of CS1 peptides to their receptor (VLA-4) on CD4 cells. To confirm this, we performed cell binding studies utilizing CS1- or FN-coated plates. However, probably due to poor binding of freshly isolated T cells to such plates, appreciable binding of CD4 cells could not be observed. Therefore, we performed cell adhesion assays using the Jurkat T cell line, which consists of a mature T

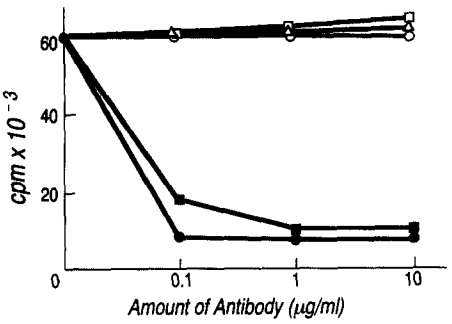


**Figure 4.** Anti-VLAβ1 and anti-VLA-4 antibodies inhibit CD4 cell proliferation induced by anti-CD3 plus CS1 peptide. CD4 T cells were incubated for 30 min with various concentrations of anti-VLAβ1 (4B4, *filled circles*), anti-VLA-4 (8F2, *filled squares*), anti-VLA-5 (2H6, *open circles*; and mAb 16, *open triangles*), or anti-LFA1 (2F12, *open squares*), and then plated on culture plates coated with anti-CD3 plus CS1 peptide. After 4 d of culture, proliferation was assessed by determination of [³H]thymidine incorporation. The data are expressed as the mean of triplicate samples. Each SD was <15%. The data shown are representative of five separate experiments.

cell population expressing CD3/TCR, CD4, VLA-4, and VLA-5 antigens (data not shown).

As shown in Fig. 5 *a*, Jurkat cells hardly bound to control CS1-uncoated plates. Their binding increased >20-fold when they were plated in microtiter plates coated with CS1-IgG conjugates. On the other hand, CS1-scrl showed no binding activity, suggesting that the binding of Jurkat cells to CS1 peptides is specific. When Jurkat cells were preincubated with a saturating amount of anti-4B4, binding of CS1 coated plates was almost completely abolished (Fig. 5 *b*). Likewise, anti-8F2 (anti-VLA-4) inhibited this binding, although its effect was partial compared with anti-4B4. In contrast, neither anti-2H6 nor mAb 16 (Fig. 5 *b*), nor other anti-VLA antibodies (data not shown), showed inhibitory activity. Thus, only anti-4B4 and anti-8F2 inhibited binding, suggesting that the binding of Jurkat cells to CS1 is mediated by VLA-4.

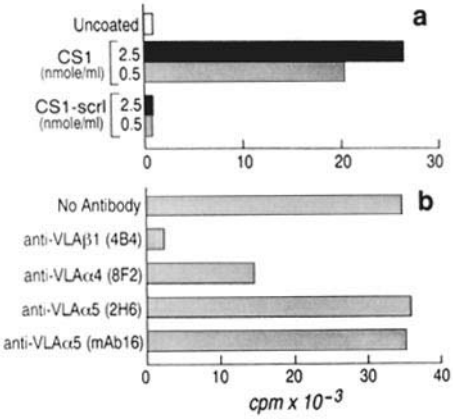


**Figure 5.** VLA-4 mediates Jurkat cell binding to CS1-coated plates. (*a*) Jurkat cells specifically adhere to plates coated with CS1 peptide. Adhesion assays were performed as described in Materials and Methods using microtiter plates coated with CS1 or CS1-scrl peptide at the indicated doses. Binding is expressed as counts per minute by [³H]thymidine incorporated into bound cells. (*b*) Anti-VLAβ1 and anti-VLA-4 antibodies block Jurkat cell binding to a CS1-coated plate. Jurkat cells were incubated with indicated antibodies (10 μg/ml) for 30 min before the adhesion assays using microtiter plates coated with CS1 peptide (2.5 nmol/ml). Background binding to CS1-uncoated plates was <1,000 cpm.

*Activity of CS1 Peptides for Promoting CD4 Cell Proliferation Parallels Their T Cell Adhesion-Promoting Activity.* To further determine whether the adhesion activity of various CS1-related peptides for Jurkat cells and their activity in promoting CD4 cell proliferation were correlated, we compared Jurkat T cell attachment activity and CD4 cell stimulation activity between CS1 and its peptide homologues listed in Fig. 1. As shown in Fig. 6 *a*, the original CS1 peptide had the strongest Jurkat cell binding activity, followed by CS1-D, and then CS1-A. This order of adhesive activity exactly matched the results seen using a melanoma cell line (Komoriya et al., manuscript in preparation). Furthermore, this was also the same order seen in their activity to stimulate T cells, with CS1 being the strongest, CS1-D second, and CS1-A third (Fig. 6 *b*). CS1-B and CS1-scrl had neither adhesion nor activation activities. These results, taken together with the antibody inhibition studies, strongly suggest that the binding of CS1 peptide by CD4 cells via VLA-4 is required for their proliferation in our system. Moreover, our results suggest that the EILDVPST sequence in the CS1 domain appears to be significantly involved in both the T cell adhesion and activation processes.

*CD4 Cell Proliferation Induced by Anti-CD3 and Native Plasma FN Is Dependent on Both VLA-4 and VLA-5.* Based on the above findings, we concluded that the CS1 alternatively spliced domain of FN, through its interaction with VLA-4, provides a signal synergistic with anti-CD3 to promote CD4 cell proliferation in this serum-free culture system. Next, we determined whether the CS1 domain within the native FN molecule could act in a fashion similar to these synthetic CS1 peptides in inducing CD4 cell activation. For this

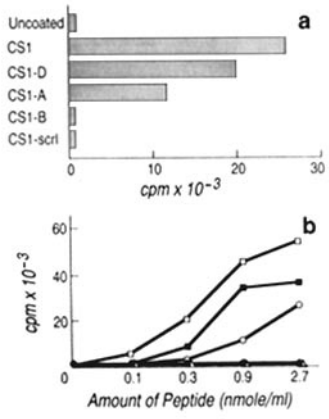


**Figure 6.** The activity of CS1 in promoting CD4 cell proliferation parallels its strength of T cell adhesion promoting activity. (*a*) Comparison of activity of various CS1 peptides to promote Jurkat cell adhesion. Adhesion assays were performed using microtiter plates coated with CS1 or its related peptides at a concentration of 2.5 nmol/ml. (*b*) Comparison of the activity of various CS1 peptides to promote CD3-dependent CD4 cell proliferation. CD4 cells were cultured on plates coated with various amounts of CS1 and its related peptides; CS1 (*open squares*), CS1-D (*filled squares*), CS1-A (*open circles*), CS1-B (*filled circles*), CS1-scrl (*open triangles*). After 4 d of culture, proliferation was assessed by determination of [³H]thymidine incorporation. The data are expressed as the mean of triplicate samples. Each SD was <15%. The data shown are representative of three separate experiments.

purpose, we performed the CD4 cell proliferation assay using purified plasma FN in place of CS1 conjugates as described previously (9).

Plasma FN includes both the CS1 domain, whose receptor was shown to be VLA-4, as well as a cell binding domain that contains the RGDS sequence, which is recognized by VLA-5. Our previous studies (9) demonstrated that anti-VLA$\beta$1 (4B4), as well as anti-VLA-5 (mAb 16), almost completely inhibited CD4 cell proliferation induced by anti-CD3 plus plasma FN. In this study, we examined CD4 cells from eight different donors to determine which FN receptors were used in the CD4 cell proliferation induced by intact plasma FN. In each of the eight donors examined, CD4 cell proliferation could be inhibited >95% by adding either anti-VLA$\beta$1 or anti-VLA-5 (mAb 16 or 2H6) antibodies to the culture medium at a concentration of 0.4 $\mu$g/ml or more. This supported the previous data that CD4 cells predominantly use VLA-5 as FN receptor in anti-CD3 plus FN-induced activation. Fig. 7 shows representative data obtained from two donors. Although the inhibition by anti-VLA-5 was predominant, it should also be noted that anti-VLA-4 (8F2) exhibited a significant but partial inhibitory effect. The inhibition of VLA-4-mediated proliferation by 8F2 ranged from 10 to 40% (mean 33%) in eight donors. On the other hand, anti-LFA1 (2F12) antibody had no effect, confirming the specificity of the inhibition by anti-8F2. Separate experiments showed that soluble CS1 peptide could also partially inhibit CD4 cell activation in response to intact plasma FN plus anti-CD3 (data not shown). These studies suggest that T cell proliferation induced by native FN plus anti-CD3 is dependent on both VLA-4 and VLA-5, although the VLA-5 receptor appears to predominate.

*Interaction of VLA-5 with RGDS May Be Required for VLA-4/CS1-mediated CD4 Cell Proliferation Induced by Anti-CD3 Plus Plasma Fibronectin.* Although anti-VLA-4 inhibited CD4 cell proliferation by 10–40% in our system, nearly complete inhibition can be achieved by anti-VLA-5. It is unlikely, however, that anti-VLA-5 crossreacts with VLA-4 or that perturbation of the VLA-5 molecule by anti-VLA-5 antibody transmits a negative signal to the VLA-4/CS1 pathway, since anti-VLA-5 had no effect on CD4 cell proliferation induced by the CS1 peptide (Fig. 5). Therefore, the above findings imply that the VLA-5 receptor may play a role in facilitating the interaction of the CS1 domain in native FN with the VLA-4 receptor. Our previous observation that addition of RGDS peptides almost completely blocks CD4 cell proliferation induced by anti-CD3 plus plasma FN (9) supports the notion that interruption of interaction of the VLA-5 with the RGDS-containing domain results in an inability of the VLA-4/CS1 complex to function. To further clarify the above points, we used a unique anti-FN antibody, mAb 333. This antibody binds close to central cell binding domain, and thus, interferes with binding of the RGDS domain to the VLA-5 receptor (20). Moreover, it has been recently shown that mAb 333 does not block adhesion between the CS1 region of native FN and VLA-4 (12). Therefore, we tested whether the CS1 domain could induce CD4 cell activation after the interaction between RGDS sites and VLA-5 was blocked by mAb 333. As shown in Fig. 7, virtually no T cell proliferation was observed when mAb 333 was added to the culture system at a concentration of 2 $\mu$g/ml or more. This result strongly suggests that the interaction of VLA-4 and CS1 does not operate efficiently when the RGDS domain of native plasma FN is blocked. Thus, the interaction of VLA-5 with RGDS may be required to facilitate the interaction between CS1 and the VLA-4 receptor for CD4 cell proliferation, or the VLA-4 and VLA-5 receptors may undergo a partial cooperativity in their interaction with FN.

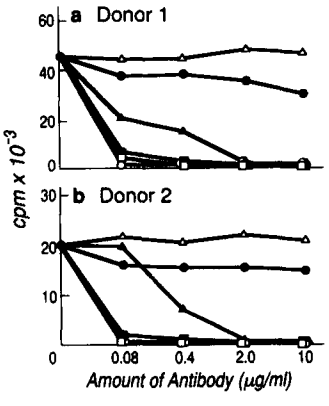


**Figure 7.** CD4 cell proliferation induced by anti-CD3 plus native plasma FN is dependent on both VLA-4 and VLA-5, but this activation does not occur after the VLA-5/RGDS interaction is blocked by either anti-VLA5 or mAb 333. CD4 cells were preincubated with the indicated amount of various antibodies for 30 min: anti-VLA$\beta$1 (4B4; *open circles*), anti-VLA-4 (8F2, *filled circles*), anti-VLA-5 (2H6, *open squares*, and mAb 16, *filled squares*), and anti-LFA1 (2F12, *open triangles*), and then cultured on plates coated with anti-CD3 (0.1 $\mu$g/ml) plus plasma FN (10 $\mu$g/ml). mAb 333 (*filled triangles*) was added to the culture plates 30 min before addition of CD4 cells. After culturing for 4 d, proliferation was assessed by determination of [$^3$H]thymidine incorporation. The data are expressed as the mean of triplicate samples. Each SD was <15%. Representative data from two (*a*, donor 1; and *b*, donor 2) of eight donors are shown.

## Discussion

Using synthetic CS1 peptide, we have shown that this alternatively spliced domain of FN promotes not only T cell adhesion, but also CD4 cell proliferation when combined with anti-CD3 in a serum-free culture system. Moreover, these two different biologic processes, i.e., adhesion and proliferation, were shown to be mediated by the VLA-4 receptor. In addition, our study showed that the CD4 cell proliferation induced by anti-CD3 plus native plasma FN is dependent upon both VLA-4 and VLA-5, although the latter is predominant. These results might help explain the preferential responsiveness of CD4$^+$CD45RA$^-$CD29$^+$(CD29$^{high}$) cells to soluble antigens (4, 22), since this CD4 cell subset expresses high levels of both the VLA-4 and VLA-5 FN receptors (9, 23), which can synergize with the CD3/TCR pathway during T cell activation.

It is clear that synthetic CS1 peptide provides a potent costimulus for CD4 cells. However, when the CS1 domain is located within native plasma FN molecules, the CS1/VLA-4 interaction seems to be less active in its ability to generate an activation signal for CD4 cells than the VLA-5/RGDS interaction. Although CD4 cell proliferation in response to native FN plus anti-CD3 was partially inhibited by anti-VLA-4 antibodies, proliferation was essentially completely inhibited when the VLA-5/RGDS interaction was blocked by anti-

VLA-5, RGDS peptide, or mAb 333. Our results suggest that VLA-4-mediated proliferation is dependent upon interaction of VLA-5 with the RGDS domain contained in the native plasma FN molecule. Hemler et al. (24) have also reported adhesion data quite similar to our own, obtained using K562 cell lines transfected with VLA$\alpha$4 cDNA. Although the transfected K562 cells expressed both VLA-4 and VLA-5 in approximately equal levels, their adhesion to intact plasma FN was almost completely inhibited by anti-VLA-5, but not by anti-VLA-4. On the other hand, our present data contrast with previous reports that VLA-4 and -5 can independently recognize the CS1 and RGDS domains of native FN, respectively (10, 12). Mould et al. (12) have recently demonstrated that spreading of A375-M, a human metastatic melanoma cell line, could be observed even after the central cell binding domain (RGDS) was blocked by anti-mAb 333, and that the residual spreading could be completely inhibited by either anti-VLA$\beta$1 or anti-VLA-4. These adhesion data indicate that the CS1 domain in native FN is active and can promote cell adhesion independently from the RGDS domain. The apparent discordance between our results and the previous binding studies could be due to methodological differences (in proliferation and adhesion assays), or due to the different cell types used (peripheral CD4 cells vs. adherent cell lines).

There may be several possible explanations for the apparent requirement for VLA-5/RGDS interaction in VLA-4/CS1-dependent activation of CD4 cells by anti-CD3 plus native FN. First, the binding affinity of VLA-4 on resting CD4 cells for the CS1 domain might be quite low (presumably lower than the same receptor on melanoma cells), so it would not be able to bind FN. According to this hypothesis, the VLA-5/RGDS interaction might facilitate the binding of VLA-4 simply by stabilizing the CS1/VLA-4 interaction, or by activating VLA-4 to a high-avidity state. A second possibility is that the binding of VLA-5 changes the conformation of the FN molecule, permitting more effective binding of VLA-4 to the CS1 site. A third possibility is that the amount of CS1 domain contained in plasma FN may not be enough to activate CD4 cells without assistance from the RGDS/VLA-5 interaction. Plasma FN is composed of two polypeptide chains (25) covalently associated to form a dimer. Both chains have central cell binding domains containing RGDS sites, so each plasma FN molecule has two binding sites for VLA-5. In contrast, only one chain has the IIICS containing the CS1 sequence. Moreover, the CS1 sequence can be spliced out of some FN molecules. Therefore, the molar ratio of CS1 and RGDS in human plasma FN is <1:2. This lower ligand density may lead to the inability of the CS1/VLA-4 interaction to activate CD4 cells by itself. To increase the density of CS1, FN was coated onto the plates at a higher concentration (up to 100 $\mu$g/ml). However, mAb 333 was still able to completely abolish CD4 cell proliferation (data not shown). Although we cannot exactly compare the number of CS1 sequences contained in plasma FN with those in the synthetic peptide-IgG conjugates we used, the latter may be better able to trigger VLA-4-mediated activation because of their comparatively greater ligand density. Whatever the mechanism, it is clear that peripheral CD4 cells preferentially use VLA-5 as functional FN receptor in our CD4 cell activation system. This finding should be stressed, considering the fact that VLA-4 is expressed more abundantly than VLA-5 on peripheral CD4 cells (7).

The predominant usage of VLA-5 by CD4 cells does not necessarily negate the significance of VLA-4 in T cell function. At present, we can only use plasma and not cellular FN as a source of FN. Since a cell type–specific difference in the CS1 expression has been demonstrated (26), it may be possible that the CS1 domain of FN is more abundantly expressed in tissue matrix, inflammatory regions, or on the surface of APC. If such were the case, FN might have a higher avidity for binding T cells bearing VLA-4 (11). Furthermore, it has been recently demonstrated that VLA-4 is also a receptor for VCAM-1, which is a cell surface adhesion molecule in the Ig gene super family (27). VCAM-1 can be induced on lymphokine-activated endothelial cells (28). Using K562 cell lines transfected with VLA-$\alpha$4 cDNA, Elices et al. (27) have shown that VCAM-1 interacts with VLA-4 at a site distinct from the CS1 binding site. Most recently, Komoriya et al. (manuscript in preparation) have narrowed down the minimum active site for melanoma cell adhesion to CS1 to a tripeptide, Leu-Asp-Val (LDV). Most interestingly, the LDV sequence can be found in VCAM-1 (28). If this sequence is also active in VCAM-1 as in FN, the interaction of VLA-4-bearing T cells with VCAM-1 on activated endothelial cells could result in T cell activation. Although further studies are necessary to determine whether the LDV sequence in VCAM-1 plays a role in CD4 cell activation, the above result leads us to speculate that VLA-4 may play a key role in inflammatory sites through interactions with activated endothelium or FN deposited within these sites (27).

Evidence that adhesion molecules play an important role in T cell immune function has been accumulating (29). The primary pathway for clonal expansion of T cells occurs by means of antigen recognition by the CD3/TCR complex. However, a number of cell surface adhesion molecules, including CD2/LFA-3 (30–32), CD4/MHC class II (33, 34), CD8/MHC class I (35), and LFA-1/ICAM-1 (36), have been shown to synergize with the CD3/TCR complex in this antigenic response. Cell-cell contact may be an important consequence of these adhesion events; however, these accessory interactions also appear to trigger intracellular signals that modify antigen-driven proliferation via the CD3/TCR complex (36, 37). In addition to cell-cell interactions, cell-extracellular matrix protein contacts have attracted increasing attention by virtue of their costimulatory effect upon lymphocyte activation (9, 38, 39). Dang et al. (38) have recently demonstrated that collagen provides a costimulatory signal with the CD3 pathway in promoting CD4 cell proliferation. This effect is mediated by VLA-3 and CD26 (1F7). Moreover, laminin has also been shown to induce CD3-dependent T cell proliferation via interaction with VLA-6 (39). Thus, a costimulatory effect on lymphocyte activation may be a general characteristic of extracellular matrix proteins.

Little is known about the mechanism of comitogenic signals induced by extracellular matrix proteins. It is also still unclear why mammalian cells have multiple specificities in their

ligand-receptor interactions, such as FN/VLA-4 vs. FN/VLA-5 and VLA-4/FN vs. VLA-4/VCAM-1. Are there any differences in the signal transduction and biological consequences caused by stimulation of these various ligand-receptor combinations? Engagement of these receptors by their respective ligands generates intracellular signals that lead ultimately to the biological expression of distinct functional programs of each T cell subset. The T cell proliferation system described in our study is a suitable model for continued study of the intracellular signals delivered to T cells by extracellular matrix proteins.

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