

Ligation of VLA-4 on T Cells Stimulates Tyrosine Phosphorylation of a 105-kD Protein

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Summary

The VLA/integrins are a family of heterodimeric adhesion receptors shown to be involved in cell-to-cell and cell-to-extracellular matrix (ECM) interactions. Given recent evidence that VLA molecules can synergize with the CD3/T cell receptor (TCR) pathway to activate T cells, it is important to identify biochemical event(s) generated by these molecules. Here, we report that the engagement of VLA-4 on T cells with specific antibodies or its ligand activates protein-tyrosine kinase (PTK) activity as detected by antiphosphotyrosine immunoblotting. The crosslinking of VLA- β 1 (CD29) with a specific monoclonal antibody (mAb) (anti-4B4) plus anti-mouse immunoglobulin resulted in the rapid tyrosine phosphorylation of a 105-kD protein (pp105) in the human T cell line H9, as well as in peripheral resting T cells. The increase in tyrosine phosphorylation of pp105 was specifically mediated by VLA-4, since mAbs against α 4, but not against other VLA α chains, could induce this phosphorylation. In addition, the binding of T cells with the CS1 alternatively spliced segment of fibronectin (the binding site recognized by VLA-4) induced pp105 tyrosine phosphorylation. Crosslinking the CD3 complex or VLA-4 molecules with mAbs demonstrated that each of these molecules stimulated the tyrosine phosphorylation of unique sets of proteins with different kinetics, suggesting that these two receptor systems are coupled to distinct PTKs. Since tyrosine phosphorylation of cellular proteins has been shown to be a crucial biochemical event in cell growth, our findings suggest that the induction of pp105 tyrosine phosphorylation via VLA-4 may play a role in the transduction of activation signals through this molecule.

Adhesion molecules on T cells have attracted increasing attention because of their diverse roles in differentiation, recirculation, antigen recognition, activation, and proliferation (1, 2). According to their basic structure, lymphocyte adhesion molecules have been classified into three broad categories: the immunoglobulin, integrin, and selectin families (1). The VLA antigens constitute the β 1 integrin subfamily (3), and are one of the major adhesion receptors expressed by T cells. They are transmembrane glycoproteins consisting of a common β 1 subunit (CD29) noncovalently associated with distinct α chains to form different heterodimers (2). The VLA proteins function as cell surface receptors mediating cell-to-cell and cell-to-extracellular matrix protein (ECM)¹ adhesive interactions. On resting CD4⁺ T cells, the VLA/CD29 antigens are preferentially expressed on the CD45RO⁺CD45RA⁻ helper/inducer (memory) subset (4, 5). Presumably, VLA molecules allow these helper cells to interact effectively with the surrounding ECM or aid migra-

tion into tissues. Recent studies have clearly shown that the VLA molecules not only mediate T cell adhesion, but can synergize with the CD3/TCR pathway to promote T cell proliferation (6–11). Coimmobilization of ligand proteins for the VLA receptor family and submitogenic doses of anti-CD3 results in strong T cell proliferation through at least five receptor/ligand interactions: VLA-3/collagen, VLA-4/fibronectin (FN) (CS1 region), VLA-4/VCAM-1, VLA-5/FN (RGD sequence), and VLA-6/laminin. Furthermore, it has been proposed that the expression of FN receptors (VLA-4 or VLA-5) on a subpopulation of thymocytes plays a role in thymic differentiation (12, 13). While the interaction of VLA molecules with ECM or cell surface molecules may have a variety of biological effects on T cells, the nature of the signals generated by the engagement of the VLA with their ligands is still far from clear.

Recent studies have documented that tyrosine phosphorylation on cellular proteins is an early and obligatory event in the activation of T cells (14–20). Ligation of the CD3/TCR complex by anti-CD3 antibody induces rapid tyrosine phosphorylation of several intracellular proteins, including the CD3-associated ζ chain (15, 16) and phospholipase C (PLC)-

¹ Abbreviations used in this paper: ECM, extracellular matrix; FN, fibronectin; PLC, phospholipase C; PTK, protein-tyrosine kinase; RSV, Rous sarcoma virus.

$\gamma 1$ (17, 18), which precedes the increase in inositol-1, 4, 5-trisphosphate and cytosolic Ca^{2+} concentrations. Moreover, protein-tyrosine kinase (PTK) inhibitors can prevent TCR/CD3-mediated PLC activation and cytosolic Ca^{2+} influx (19, 20). These results suggest that PTKs are closely associated with signaling through the TCR/CD3 complex and regulate subsequent events that ultimately lead to T cell proliferation. It is not known which PTKs are responsible for these early phosphorylation events. However, $p56^{lk}$ and $p59^f$ have been shown to be associated with CD4/CD8 (21, 22) and the TCR/CD3 complex (23), respectively, and are considered to be likely candidates. There also have been reports (24–27) suggesting that PTK activation may play a role in the cell adhesion process. In Rous sarcoma virus (RSV)-transformed cells, the FN receptor (integrin), as well as several cytoskeletal proteins that are closely linked to FN receptors, were shown to be hyperphosphorylated on tyrosine residues (24). In addition, it was demonstrated that several src family PTKs such as *c-yes*, *c-src*, and *c-lyn* were enriched in cell-to-cell adhesion junctions in normal adult tissues where the level of tyrosine phosphorylation is elevated (27). However, direct evidence showing that PTK activation is functionally associated with signal transduction through the VLA molecules is lacking.

Given the costimulatory signals delivered when VLA and CD3 antigens are simultaneously triggered, we examined whether the perturbation of VLA molecules could activate tyrosine kinases. We report here that engagement of VLA molecules by specific antibodies, or by their ligands, rapidly stimulated tyrosine phosphorylation on a 105-kD protein (pp105) in both the H9 T cell line and peripheral resting T cells. The induction of pp105 tyrosine phosphorylation appears to be specific for the VLA-4 molecule. Finally, PTK activation stimulated via VLA-4 was regulated independently from that associated with T cell activation through the CD3/TCR pathway.

Materials and Methods

mAbs and Reagents. mAbs reactive with CD3 (RW24B6, IgG2b), CD11a/LFA-1 (2F12, IgG1), CD20 (B1, IgG2a), CD29/VLA- $\beta 1$ (4B4, IgG1), CD45 (GAP 8.3, IgG2a), CD56 (NKH1, IgG1), MHC class I (W6/32, IgG2a), and MHC class II (9-49, IgG2a), were used in our study. Their production and characterization have been described elsewhere (4, 28–30). Anti-CDw49a/VLA-1 (TS2/7, IgG1) was kindly provided by Dr. Martin E. Hemler (Dana-Farber Cancer Institute, Boston, MA). mAbs against CDw49b/VLA-2 (G19, IgG1) and CDw49f/VLA-6 (GoH3, rat IgG2a) were obtained from AMAC, Inc. (Westbrook, ME). Anti-CDw49c/VLA-3 (PIB5, IgG2a) was obtained from Oncogene Science, Inc. (Manhasset, NY). Anti-CDw49d/VLA-4 (8F2, IgG1), and anti-CDw49e/VLA-5 (2H6, IgG1) were developed in our laboratory and described previously (6, 10). Antiphosphotyrosine antibody (4G10, IgG2b) and genistein were purchased from Upstate Biotechnology, Inc. laboratories (Lake Placid, NY). Affinity-purified goat anti-mouse Ig was obtained from Tago, Inc. (Burlingame, CA). Human FN was obtained from Telios Pharmaceuticals, Inc. (San Diego, CA). BSA, transferrin, and soybean lipids used in the serum free medium were from Boehringer Mannheim Biochem-

icals (Mannheim, Germany). IMDM, Tyr(P), Ser(P), and Thr(P) were from Sigma Chemical Co. (St. Louis, MO). The synthetic CS1 peptide (CDELPQLVTLPHNHLGPEILDVPST) that contains residues 1–25 of the type III connecting segment of FN and its rabbit IgG-conjugated form (31) was kindly provided by Dr. Martin J. Humphries (Manchester University, England), and Dr. Kenneth M. Yamada (National Institute of Dental Research, Bethesda, MD).

T Cells. The human T cell lines H9, Jurkat, HPB-ALL, and MOLT-4, were used in our study. Cell lines were cultured in RPMI 1640 containing 10% heat-inactivated FCS, and 2 mM glutamine. PBMC were separated from whole blood by centrifugation over Ficoll-Hypaque. Monocytes were depleted by adherence to plastic dishes for at least 1 h. Nonadherent cells were then depleted of B cells, NK cells, and residual monocytes with negative selection with anti-CD20, anti-Ia, and anti-CD56 using goat anti-mouse immunomagnetic Dynabeads M-450 (Dynal Inc., Great Neck, NY). The resulting cells were used as resting T cells.

Stimulation of Cells and Preparation of Cell Lysates. Cells were washed three times and resuspended in Iscove's serum-free media and dispensed into 1.5-ml Eppendorf tubes with $2.5\text{--}5.0 \times 10^6$ cells/1 ml/sample. The samples were either left as controls or incubated with saturating concentrations of mAb (10 $\mu\text{g/ml}$) for 15 min at 4°C, washed once with cold media, and then incubated with 200 μl of media containing anti-mouse Ig (10 $\mu\text{g/ml}$) at 37°C for different time periods. The reactions were terminated by the addition of 1 ml of stop solution (cold PBS containing 5 mM EDTA, 10 mM NaF, 10 mM Na pyrophosphate, and 0.4 mM Na vanadate). Cells were pelleted and then solubilized in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris HCl, pH 8.0, 5 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 10 mM NaF, 10 mM Na pyrophosphate, and 0.4 mM Na vanadate) for 15 min on ice. After removing insoluble material by centrifugation at 14,000 rpm for 10 min, supernatants were subjected to electrophoresis according to the method described below. In some experiments, cells were stimulated with CS1 peptide/rabbit IgG conjugate immobilized on six-well cultured plates (Costar Corp., Cambridge, MA). Preparation of plates coated with the CS1/IgG conjugate was described previously (10). Cells were cultured in these wells for the period indicated, followed by addition of cold stop solution, and then solubilized by lysis buffer as described above.

Electrophoresis and Immunoblotting. Cell lysates were separated on a 7.5% SDS-polyacrylamide gel under reducing conditions according to the method of Laemmli, (31a) and then electrophoretically transferred to nitrocellulose membrane, and blotted using ^{125}I -labeled antiphosphotyrosine antibody (αPT) using methods described elsewhere (32). Affinity-purified αPT was iodinated to a specific radioactivity of 10–20 $\mu\text{Ci}/\mu\text{g}$ protein using Iodobeads (Pierce Chemical Co., Rockford, IL). Autoradiographic exposures were performed using Kodak XAR film (Eastman Kodak, Rochester, NY).

Results

Crosslinking VLA Molecules with anti-CD29 mAb Stimulates Tyrosine Phosphorylation of a 105-kD Protein in the H9 T Cell Line. We have previously shown that crosslinking VLA molecules with anti-VLA- $\beta 1$ (anti-CD29) antibody, as well as immobilized FN, are comitogenic in CD3-dependent CD4 $^{+}$ cell proliferation (33). Therefore, to examine whether ligation of VLA molecules had an effect on tyrosine phosphory-

lation, we compared the patterns of tyrosine phosphorylation of several T cell lines before and after the crosslinking of VLA molecules with the anti-CD29 mAb, anti-4B4. T cells were incubated with anti-CD29 and then incubated with prewarmed (37°C) media containing goat anti-mouse Ig for different time periods (1–60 min). Cell lysates from anti-CD29-treated and untreated cells were run on SDS-PAGE, and proteins phosphorylated on tyrosine residues were detected by immunoblotting with α PT. As shown in Fig. 1, anti-CD29 induced a significant increase in the tyrosine phosphorylation of a protein with an apparent molecular mass of 105 kD (pp105) in H9 cells. A faint increase in tyrosine phosphorylation on pp105 could be seen 1 min after anti-CD29 crosslinking, and reached a maximal level after 5–10 min. The level of phosphorylation declined slightly after 30–60 min, but was still significantly increased for at least 3 h (data not shown). Because of the high level of tyrosine phosphorylation after anti-CD29 crosslinking, pp105 was readily detectable after relatively brief autoradiographic exposures (1–2 h). Under these conditions, pp105 was the only tyrosine-phosphorylated protein observed in H9 cell lysates (Fig. 1). Occasionally, pp105 appeared to be a doublet, with the appearance of a faint additional band at a slightly higher position. Other phosphorylated proteins became visible after prolonged exposure. However, no difference in the degree of tyrosine phosphorylation of these bands was observed before and after treatment with anti-CD29. Despite the high degree of CD29 expression on a number of other T cell lines including Jurkat, HPB-ALL, and MOLT-4, only H9 cells exhibited phosphorylation of this 105 kD molecule after anti-CD29 crosslinking (data not shown).

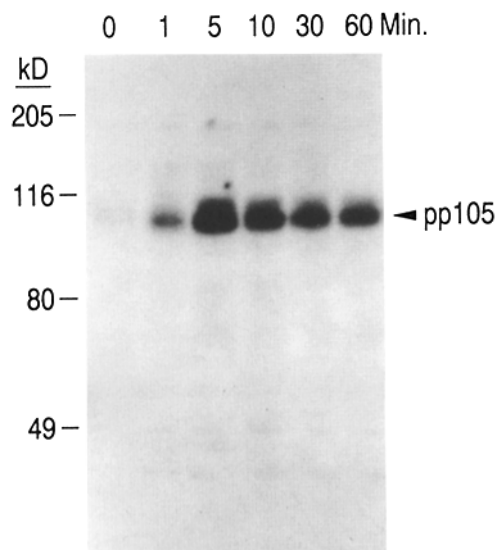


Figure 1. Crosslinking VLA molecules with anti-VLA- β 1 (anti-CD29) stimulates tyrosine phosphorylation of pp105 in H9 cells. H9 cells were treated for the time indicated (min) with anti-CD29 (4B4, 10 μ g/ml), and crosslinked with anti-mouse Ig. Proteins phosphorylated on tyrosine residues were detected by immunoblot analysis using 125 I-labeled α PT antibody (4G10).

Next, we examined whether the induction of tyrosine phosphorylation of pp105 was specific for anti-CD29. As shown in Fig. 2, although anti-CD29 crosslinking clearly induced pp105 tyrosine phosphorylation in H9 cells (lanes 1 and 2), the crosslinking of control antibodies against surface molecules with similar levels of expression, including LFA-1 (2F12), MHC class I molecule (W6/32), and CD45 leukocyte common antigen (GAP 8.3), failed to stimulate pp105 phosphorylation (lanes 3–5). Anti-CD29 alone, without anti-mouse Ig, elicited a minimal response (lane 6), suggesting that crosslinking or coclustering of VLA antigens was necessary for the induction of pp105 tyrosine phosphorylation. To further confirm the specificity of our observation, we showed that preincubation of the immunoblot with excess amounts of phosphotyrosine (10 μ M), but not phosphoserine or phosphothreonine, blocked the detection of pp105 (Fig. 3 A). In addition, pretreatment of cells with genistein, a potent PTK inhibitor, inhibited pp105 phosphorylation in a dose-dependent fashion (Fig. 3 B). The viability of H9 cells was not affected over this range of genistein concentration. Pretreatment of cells with cycloheximide (20 μ M, 4 h) had no effect on the anti-CD29-related increase in tyrosine phosphorylation of pp105 (data not shown). These results indicated that the immunoreactive 105-kD band present after crosslinking of the VLA antigens was due to an increase in its tyrosine phosphorylation, and not to an increase in its protein synthesis. Thus, our results suggested that the crosslinking of VLA antigens with anti-CD29 could increase tyrosine phosphorylation of pp105 in the H9 T cell line.

VLA-4 Is Involved in the Induction of pp105 Tyrosine Phosphorylation in H9 Cells. To clarify which of the VLA proteins is involved in the tyrosine phosphorylation of pp105, we performed the same crosslinking studies using mAbs specific for each of the VLA- α chains. Immunofluorescence analysis revealed that H9 cells expressed VLA-3 (40.1%), -4 (99.1%), -5 (22.2%), and -6 (78.2%), and the expression of VLA-1 and -2 were minimal (7.8%, and 0.6%, respectively; data not shown). However, as shown in Fig. 4, of the 6 different VLA α chain-specific antibodies, only anti-VLA-4 (8F2) could increase tyrosine phosphorylation on

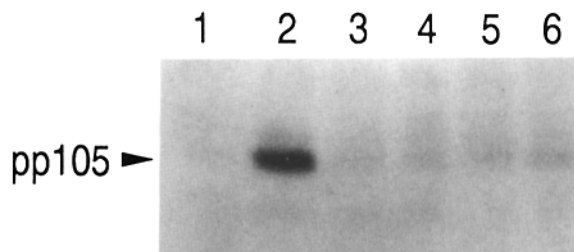


Figure 2. Induction of pp105 tyrosine phosphorylation is specific for anti-CD29. H9 cells were incubated with medium (lane 1), anti-CD29 (10 μ g/ml, lane 2), anti-LFA-1 (10 μ g/ml, lane 3), anti-MHC class I (10 μ g/ml, lane 4), and anti-CD45 (10 μ g/ml, lane 5), followed by crosslinking with anti-mouse Ig for 10 min at 37°C. H9 cells were also treated with anti-VLA- β 1 alone without crosslinking by anti-mouse Ig (lane 6). The tyrosine phosphorylation of pp105 was detected by α PT immunoblotting.

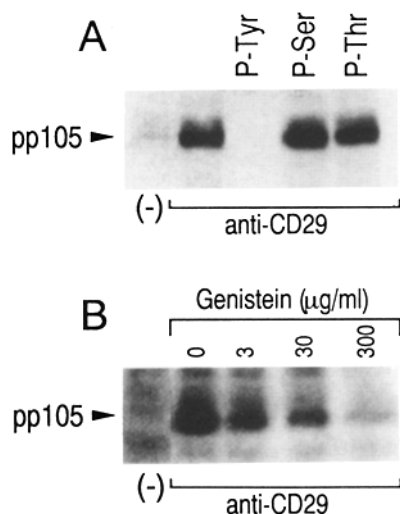


Figure 3. (A) Detection of pp105 was inhibited by phosphotyrosine, but not by phosphoserine or phosphothreonine. Cell lysates from untreated (-) or anti-CD29-treated (anti-CD29) H9 cells were separated on 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and probed with 125 I-labeled α PT in the absence or presence of phosphotyrosine (p-Tyr), phosphoserine (p-Ser), or phosphothreonine (p-Thr). (B) Induction of pp105 tyrosine phosphorylation could be inhibited by genistein. H9 cells were pretreated with genistein at the indicated concentrations for 15 min at 37°C in the presence of 1% DMSO, and then treated with anti-CD29 crosslinking. Viability of H9 cells was not affected over this range of genistein concentration. pp105 in each cell lysate was detected by α PT immunoblotting.

pp105 to a level comparable with that obtained using anti-CD29. None of the other anti-VLA- α mAbs had a significant effect. Although we cannot rule out the possibility that other VLA proteins expressed at the same levels as VLA-4 might also be capable of inducing tyrosine phosphorylation of pp105, our results indicate that VLA-4 is the primary molecule involved in triggering the tyrosine phosphorylation of this molecule in H9 cells.

The CS1 Alternatively Spliced Segment of FN Can also Induce pp105 Tyrosine Phosphorylation in H9 Cells. Next, we examined whether a ligand for VLA-4 could also induce tyrosine phosphorylation of pp105. Although both VLA-4 and -5 were initially identified as receptors for FN, it has since been shown that they recognize and bind distinct regions of the FN molecule. VLA-4 recognizes the alternatively spliced CS1 segment of the FN molecule (34–36), which is distinct

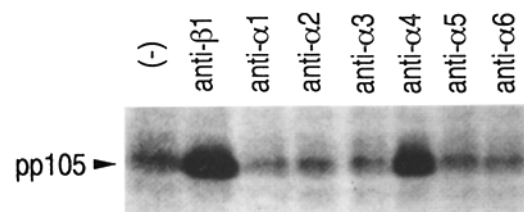


Figure 4. VLA-4 is the primary molecule involved in inducing tyrosine phosphorylation of pp105 in H9 cells. H9 cells were treated with medium alone (-) or mAbs reactive with VLA- β 1 and different VLA- α chains crosslinked with anti-mouse or anti-rat Ig. Each cell lysate was subjected to SDS-PAGE and then blotted with 125 I- α PT.

from the RGD cell binding region recognized by VLA-5 (37, 38). Therefore, we examined whether the interaction of VLA-4 with its ligand, the CS1 region of FN, could induce pp105 tyrosine phosphorylation. H9 cells specifically bound to plates coated with a synthetic CS1 peptide/IgG conjugate, but not to uncoated ones, when they were cultured on these plates for 30 min (data not shown). Under these conditions, a significant increase in the tyrosine phosphorylation of pp105 in cells from CS1-coated wells was observed (Fig. 5, lanes 1 and 2). There was a less prominent increase in tyrosine phosphorylation of the slightly higher band mentioned previously. Furthermore, the protein kinase C activator PMA, which is known to augment the binding of integrins to their ligands (5, 39), was found to upregulate the pp105 tyrosine phosphorylation induced by interaction with the CS1/IgG conjugate (Fig. 5, lane 3). PMA alone had no effect (Fig. 5, lane 4). Both the binding of H9 cells to plates coated with CS1/IgG conjugate, and the induction of pp105 tyrosine phosphorylation were almost completely blocked by the addition of soluble anti-4B4 to the culture medium (Fig. 5, lane 5). These data indicate that the interaction between VLA-4 and its ligand (the CS1 domain of FN) can trigger the tyrosine phosphorylation of pp105 in H9 T cells.

Ligation of VLA-4 with anti-VLA- α 4 Antibody or with Its Ligand Can Induce Increased Tyrosine Phosphorylation of pp105 in Peripheral Resting T Cells. We next examined whether the ligation of VLA proteins on peripheral resting T cells could stimulate tyrosine phosphorylation. As shown in Fig. 6, crosslinking VLA molecules on resting T cells with anti-CD29 induced the tyrosine phosphorylation of a protein having the same molecular weight as pp105 in H9 cells. Of six different anti- α chain antibodies, once again, only anti-VLA-4 was capable of inducing this response (Fig. 6 A). Moreover, the kinetics of phosphorylation obtained in resting

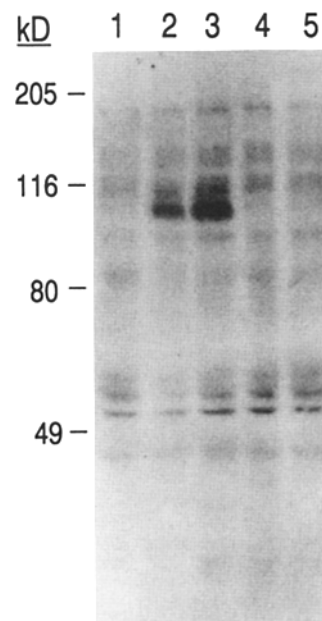


Figure 5. The binding of H9 cells with the alternatively spliced CS1 domain of FN induced tyrosine phosphorylation of pp105. H9 cells were cultured in wells coated with synthetic CS1 peptide-rabbit IgG conjugates (2.5 nmol/ml, lanes 2, 3, and 5) or uncoated wells (lanes 1 and 4), in the presence of PMA (5 ng/ml, lanes 3 and 4), or soluble anti-CD29 antibody (10 µg/ml, lane 5). After 30 min, cells were harvested, pelleted, and lysed with lysis buffer containing 1%-NP-40. Proteins phosphorylated on tyrosine residues were detected by α PT immunoblotting.

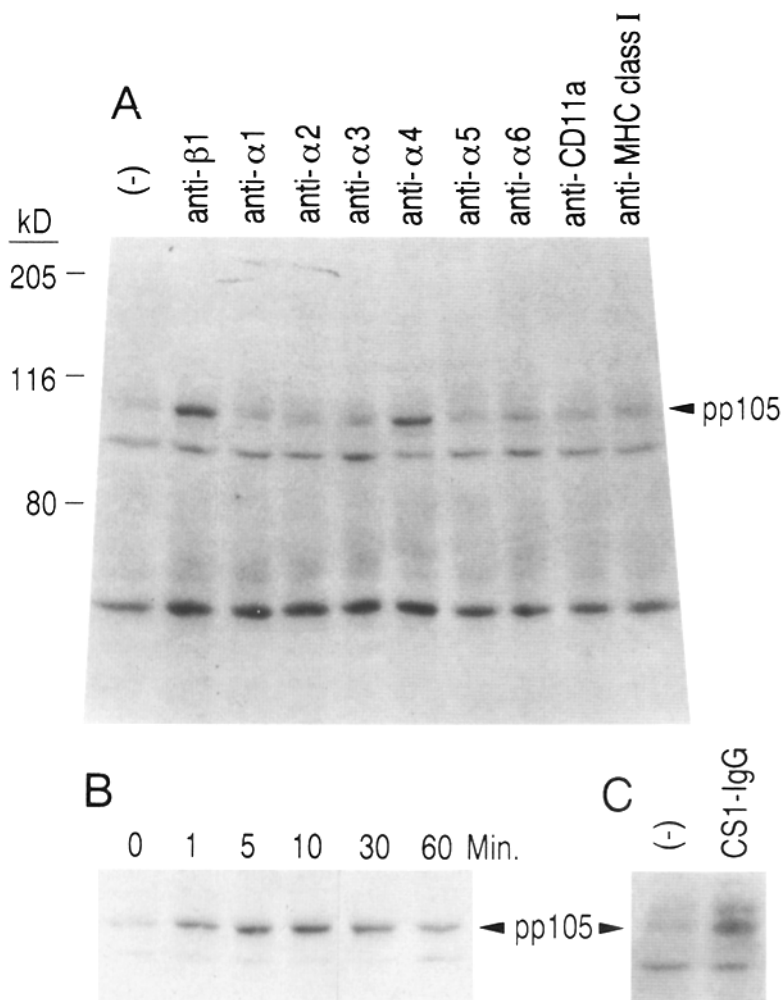


Figure 6. Ligation of VLA-4 on resting peripheral T cells also stimulated tyrosine phosphorylation of pp105. (A) Anti-VLA- β 1 and anti-VLA- α 4, but not mAbs against other VLA- α chains stimulated tyrosine phosphorylation of pp105 in resting T cells. (B) Kinetics of pp105 tyrosine phosphorylation induced by anti-CD29 crosslinking. Resting T cells were incubated with anti-CD29, followed by crosslinking with anti-mouse Ig for indicated periods (min). (C) The binding of peripheral T cells with the alternatively spliced CS1 domain of FN induced increased tyrosine phosphorylation of pp105. T cells were cultured in wells coated with synthetic CS1 peptide-rabbit IgG conjugates (2.5 nmol/ml) or uncoated wells in the presence of PMA (2 ng/ml). After 60 min, cells were harvested, pelleted, and lysed with lysis buffer containing 1%-NP-40. Proteins phosphorylated on tyrosine residues were detected by α PT immunoblotting.

T cells were exactly the same as those in H9 cells (Fig. 6 B). The same molecular mass, receptor specificity, and kinetics of phosphorylation, all suggest that the crosslinking of VLA-4 results in phosphorylation of the same molecule in both peripheral T cells and in the H9 cell line. We also attempted to examine whether immobilized CS1-IgG conjugates could induce pp105 tyrosine phosphorylation in peripheral T cells. Since resting T cells bound poorly to the CS1-IgG, we performed this experiment in the presence of PMA (2 ng/ml). A significant increase in tyrosine phosphorylation of pp105 could also be observed in T cells after their binding to the immobilized CS1-IgG (Fig. 6 C). In addition, increased tyrosine phosphorylation of the slightly higher band occurred, as seen occasionally in H9 cells. Thus, the tyrosine phosphorylation of pp105, after perturbation of the VLA-4 molecule, is not unique to the H9 cell line.

Tyrosine Kinase Activation via the CD3/TCR Pathway and the VLA-4 Molecule Are Independently Regulated. Previously, we showed (10) that VLA-4 mediates CD3-dependent T cell proliferation through interaction with its ligand, the alternatively spliced domain of FN (CS1). Since PTK activation has been shown to be an early event in T cell activation via the CD3/TCR pathway (14–23), we examined whether or

not there was a relationship between the tyrosine kinase(s) activated by the CD3/TCR and VLA-4 pathways. We compared the proteins phosphorylated on tyrosine residues after treatment of H9 cell with anti-CD3 and anti-VLA-4. As shown in Fig. 7 A, the two stimuli caused distinct patterns of increased protein tyrosine phosphorylation. In contrast to anti-VLA-4, which exclusively stimulated phosphorylation of pp105 (lane 2), anti-CD3 induced the tyrosine phosphorylation of several proteins with molecular masses of 140, 120, 116, 95, 85, and 45 kD (lane 3). In Fig. 7 B, the relative amount of tyrosine phosphorylation of pp105 (VLA-4-dependent) and pp116 (CD3-dependent) in H9 cells was graphed as a function of time after addition of each mAb. As already shown in Fig. 1, pp105 tyrosine phosphorylation induced by anti-VLA-4 reached a maximal level at 5–10 min, and persisted for at least 60 min after the initiation of stimulation with a slight decline. In contrast, the maximal level of anti-CD3-dependent pp116 tyrosine phosphorylation was attained within 1 min. By the 5-min time point, the level of phosphorylation was beginning to decline, and by 30 min it was near the basal levels. Similar kinetics were also observed for the tyrosine phosphorylation of other CD3-dependent proteins, which is consistent with previous reports

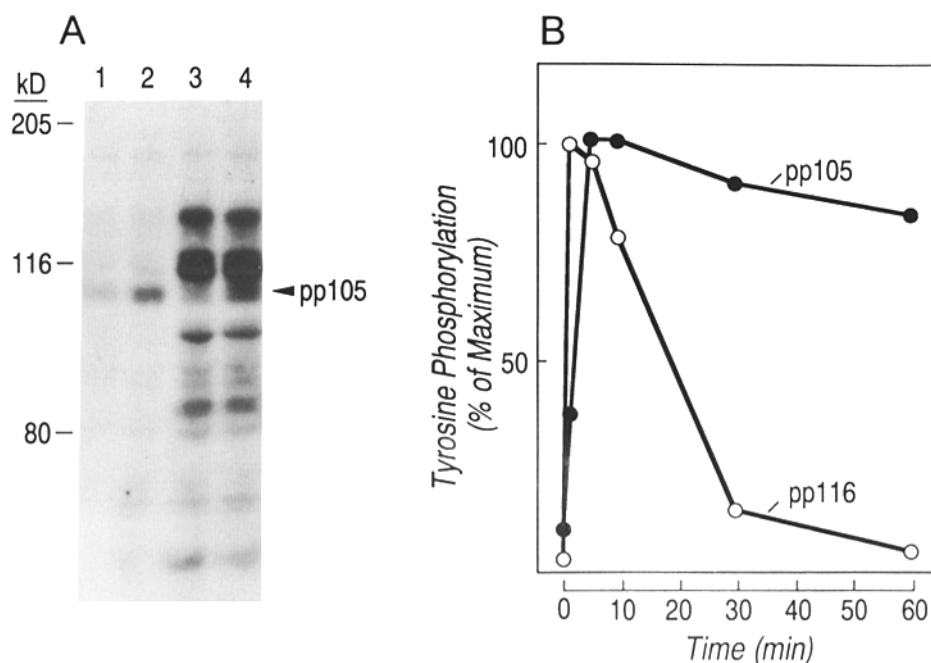


Figure 7. Tyrosine kinase activation via the CD3/TCR pathway and the VLA-4 molecule are independently regulated. (A) Crosslinking the CD3 molecule with anti-CD3 stimulated the tyrosine phosphorylation of a different set of proteins in H9 cells from that (pp105) stimulated after VLA-4 crosslinking. H9 cells were cultured in medium alone (lane 1), or treated for 1 min by crosslinking with anti-VLA-4 (lane 2), anti-CD3 (lane 3), or anti-VLA-4 plus anti-CD3 (lane 4). (B) Kinetics of tyrosine phosphorylation induced by anti-VLA-4 and anti-CD3 crosslinking. H9 cells were incubated with either anti-CD3 or anti-CD29, followed by crosslinking with anti-mouse Ig for the indicated time periods (min). The anti-CD3-induced increase in tyrosine phosphorylation of the 116-kD protein (O) and the anti-VLA-4-induced increase in tyrosine phosphorylation of the pp105 (●) were quantitated by scanning the autoradiograms with a densitometer. The peak heights are graphed as percent of maximum value.

(19, 20). Thus, the pattern and kinetics of tyrosine phosphorylation are completely different after anti-CD3 and -VLA-4 crosslinking. Furthermore, when T cells were simultaneously treated with anti-CD3 and -VLA-4, the resultant phosphorylation pattern was merely a sum of the bands obtained after stimulation with each antibody individually (Fig. 7 A, lane 4). No augmentation or reduction in the level of tyrosine phosphorylation of any of the bands was observed. The kinetics were also unaffected when cells were stimulated with a combination of both antibodies. Taken together, these results strongly suggest that the tyrosine phosphorylation, triggered via the VLA-4 molecule, is independently regulated from that stimulated via the CD3/TCR pathway. This implies that distinct tyrosine kinases might play a role in the signal transduction after ligand occupancy of VLA-4 and the TCR.

Discussion

Although considerable progress has been made in defining the structure and function of the VLA antigens, very little is known about second signals generated after ligand binding. For example, the spreading of capillary endothelial cells on FN via integrin receptors (VLA-5) can induce intracellular alkalinization resulting from activation of a transmembranous Na^+/H^+ antiporter (40). Another report (41) showed that a mAb directed against a specific epitope of the VLA- β 1 chain resulted in increased levels of intracellular cAMP in T cells stimulated by anti-CD3 or -CD2 mAb, thereby inhibiting T cell proliferation by these stimuli. However, the mechanism underlying these responses are still far from clear. In this paper, we have demonstrated that engagement of the VLA-4 molecule by specific antibodies, or by the CS1 region of FN, rapidly stimulates tyrosine phosphorylation on pp105 in resting T cells and in the H9 T cell line. Other VLA mol-

ecules (VLA-1, -2, -3, -5, and -6) did not appear to be involved in this response. In addition, the PTK activation induced by VLA-4 ligation was regulated independently from that induced by the CD3/TCR pathway. It is unclear why H9 cells are unique amongst the T cell lines tested for this signal transduction pathway. Either pp105 itself, or a PTK(s) responsible for its phosphorylation may be absent in other T cell lines. However, the significance of this pathway should be emphasized by its activation after the perturbation of VLA-4 on normal peripheral T cells. Although it still remains to be determined which PTK(s) is involved in this response, pp105 tyrosine phosphorylation appears to be a specific and early signal transmitted after the interaction of VLA-4 with external stimuli.

Since neither the β 1 nor α 4 subunits of the VLA-4 protein have inherent PTK activity, our data suggest that the VLA-4 receptor must be functionally or physically linked with a PTK(s). A putative role for PTKs in cell adhesion has been described in the past (24-27). An earlier report by Rohrschneider (24) showed that the src oncogene product, pp60^{src}, which is a tyrosine kinase, was concentrated in adhesion plaques of RSV-transformed cells. The adhesion plaque is a specialized structure for cell-to-cell and cell-to-ECM contact. Its constituent elements, including FN receptors (integrin), talin, and vinculin, were found to be hyperphosphorylated on tyrosine residues in these transformed cells. The PTK activation found in these cells was presumed to be closely associated with the transformed phenotype (25). Tsukita et al. (27) have also recently shown that proto-oncogenic tyrosine kinases such as c-yes, c-src, and c-lyn, are enriched in cell-cell adherence junctions in normal adult tissues where the level of tyrosine phosphorylation is also elevated. Thus, PTKs may be physically associated with adhesion molecules in both transformed and normal cells, and may con-

tribute to the generation of critical signals leading to a variety of biological consequences. Our present report is the first demonstration that the ligation of VLA molecules on T cells is functionally associated with PTK activation.

Thus far, we have no evidence that VLA molecules other than VLA-4 are involved in PTK activation in T cells. It is especially interesting that pp105 tyrosine phosphorylation was specific for the VLA-4 molecule, which is unique within the VLA antigen family. First, VLA-4 differs structurally from the other $\beta 1$ integrins in that the $\alpha 4$ subunit is only weakly associated with its $\beta 1$ subunit, and that the 150-kD $\alpha 4$ protein usually undergoes partial cleavage to form 80- and 70-kD fragments (42). Moreover, a 180-kD form of the $\alpha 4$ subunit, which associates with $\beta 1$, has also been identified (42). Second, the distribution of VLA-4 is relatively restricted to lymphoid and myeloid cells (2), and its expression is generally low on most adherent cells (which express other VLA molecules at higher levels). Third, although VLA-4 mediates attachment of cells to ECM like other members of the $\beta 1$ integrin family (34–36), it is also involved in cell-to-cell interactions (43). In addition to the alternatively spliced CS1 domain of FN, a second ligand has been identified for VLA-4 (43). This vascular cell adhesion molecule 1 (VCAM-1) is a member of the Ig family, and is expressed on activated endothelial cells (44). In addition, the presence of a third ligand for the VLA-4 has been postulated. This ligand is proposed to function as a counter-receptor for VLA-4 on lymphocytes, and to mediate homotypic lymphocyte aggregation triggered by mAbs that recognize a distinct epitope within the VLA molecule (45, 46). Thus, VLA-4, through its multiple ligand specificity, may confer different functional effects on T cells (47). It is presently unknown whether interactions of VLA-4 with its other ligand(s) result in pp105 tyrosine phosphorylation. However, we did observe that homotypic T cell aggregation, triggered by an anti-VLA-4 (HP2/4) antibody (kindly provided by Dr. Sanchez-Madrid, Madrid, Spain), resulted in an intense tyrosine phosphorylation of pp105 (our unpublished observation). Using a soluble VCAM-1 fusion protein, Damle and Aruffo (11) recently demonstrated that VCAM-1, like the CS1 domain of FN, could also transmit costimulatory activation signals with anti-CD3 to CD4⁺ T cells via VLA-4. It is possible that the ligation of VLA-4 by VCAM-1 may also induce pp105 tyrosine phosphorylation in T cells. If true, pp105 tyrosine phosphorylation may be a common biochemical event resulting from the ligation of the VLA-4 receptor with different ligands.

Our present study showed that pp105 tyrosine phosphorylation was independently regulated from the PTK activation that follows TCR/CD3 ligation. Meanwhile, engagement of VLA receptors on T cells with anti-VLA- $\beta 1$ did not stimu-

late turnover of phosphoinositides and had no effect on the intracellular concentration of Ca²⁺ (our unpublished observations). Each of these signals is associated with signal transduction through the CD3/TCR complex (14–23). We have shown in a previous report (48) that the engagement of the VLA molecule with FN induced the AP-1 transcriptional factor in an independent fashion from the TCR/CD3 pathway. The stimulation of the latter pathway (CD3/TCR) has been shown to induce another transcriptional factor, NFAT-1, which is felt to activate transcription of the IL-2 gene in combination with the AP-1 factor (49). In this manner, the CD3/TCR and the CD29/VLA receptor complexes appear to stimulate distinct but converging signal transduction pathways. However, previous studies have shown that at least four distinct VLA molecules (VLA-3–6) can transmit similar costimulatory signals for IL-2 production and T cell proliferation (6–11). In this regard, it remains to be shown whether pp105 tyrosine phosphorylation is actually linked to the activation of IL-2 gene transcription, or to the comitogenic proliferative signals observed after engagement of the VLA-4 molecule by its ligands (FN and VCAM-1). Whether such signals delivered to the cell via VLA-4 use other (unknown) transducing elements common to all VLA molecules is unclear. Further study of this point is underway.

In summary, our findings strongly suggest that activation of a PTK, leading to tyrosine phosphorylation of pp105, is one of the early steps in signal transduction through the VLA-4 molecule on T cells. A recent report by Kornberg et al. (50), demonstrated increased tyrosine phosphorylation of a 130 kD protein induced by clustering of $\beta 1$ integrins on human epidermal carcinoma (KB) cells. Therefore, tyrosine kinase activation after ligation of VLA molecules may be a common event found in a variety of cell types. The nature of pp105 is presently unknown. It seems to be distinct from either the $\beta 1$ subunit of VLA-4, or from vimentin, which have molecular masses of around 100 and 130 kD, respectively, and can be tyrosine phosphorylated (24). This conclusion is based on immunoprecipitation experiments where pp105 could not be precipitated with antibodies directed against each of these proteins (data not shown). It has also been shown that pp105 differs from pp100 or pp135 (19), substrates for CD3/TCR pathway-associated PTKs. Purification and/or gene cloning may be necessary for the ultimate determination of the nature and structure of pp105. Identification of the PTK(s) responsible for pp105 tyrosine phosphorylation, and the role of PTK activation in VLA-mediated effects on cell function will be the objects of future investigation. These efforts should shed more light on the molecular mechanisms of signal transduction via VLA molecules.

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