Multiple Functional Forms of the Integrin VLA-2 Can Be Derived From a Single $\alpha^2$ cDNA Clone: Interconversion of Forms Induced by an anti-$\beta_1$ Antibody

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Abstract. The integrin VLA-2 was previously found to bind to either collagen alone, or collagen plus laminin, but the mechanism for this cell-specific functional difference was unknown. Here we transfected VLA-2 $\alpha^2$ subunit cDNA into K562 cells and obtained VLA-2 (called Form-O) which bound to neither collagen nor laminin. We then used a Matrigel selection procedure to enrich for a minor subpopulation of K562 cells stably expressing a form of VLA-2 (Form-C) that bound to collagen but not laminin. In contrast, the same $\alpha^2$ cDNA transfected into RD cells yielded VLA-2 (Form-CL) which bound to both collagen and laminin. These Form-O, -C, and -CL activities were stably expressed during extended cell culture, and could not be qualitatively altered by adding phorbol esters or by exchanging the resident divalent cations. However, addition of stimulatory anti-$\beta_1$ antibodies (TS2/16, A-1A5) rapidly converted VLA-2 Form-O and Form-C into Form-CL. Anti-$\beta_1$ antibody stimulation of VLA-2 activity was observed not only on whole cells, but also with solubilized receptors. These results suggest (a) that the ligand binding specificity of VLA-2 can be determined by its cellular environment, rather than by variations in the primary sequence of the $\alpha^2$ subunit, (b) that stably inactive or partly active VLA-2 can be rapidly converted to a fully active form through conformational changes initiated at a nonligand binding site on the $\beta_1$ subunit, and (c) that the mechanisms for VLA-2 stimulation by phorbol ester and by antibody are quite distinct, because the latter does not require an intact cell.

The 20 or more distinct heterodimeric adhesion receptors in the integrin family mediate cell adhesion to an assortment of extracellular matrix and cell surface proteins (20, 27, 52). Compounding the diversity of adhesive capabilities mediated by integrins, most, if not all integrins, can assume multiple functional states. On platelets (48, 57) and leukocytes (10, 14, 51, 59, 62), several integrins can exist in a relatively nonfunctional state until triggered to rapidly become functionally active. The mechanism for this rapid (and sometimes transient) increase in integrin function on different cells in response to a host of agonists is not clear. However, protein kinase C is undoubtedly involved because phorbol esters can act downstream of specific triggering pathways to cause upregulation of integrin functions (10, 50, 61, 62). When the cytoplasmic tails of the integrin $\beta_1$ and $\alpha^1b$ chains have been deleted, the patterns of functional up-regulation for $\alpha^2b_1$ (26) and for $\alpha^1b_\beta_3$ (43) have been altered, suggesting that a connection between integrin and cytoskeletal proteins plays a critical role.

Integrin functions can be stimulated not only by phorbol esters and various other more physiologically relevant agonists, but also by certain antibodies to the integrin $\beta_1$ (4, 32, 41, 58), $\beta_2$ (29), and $\beta_3$ (42) subunits. In some of these studies, antibodies were suggested to modulate integrin affinity by directly inducing a conformational change in the receptor at the cell surface (42), but in other cases, an active cellular metabolism was required (4, 32), suggesting that the anti-integrin antibodies could possibly have an indirect effect.

In addition to having the flexibility to be turned on (and off), certain integrins also appear to undergo cell-type specific regulation of adhesive capabilities. For example, on platelets and fibroblasts, the $\alpha^2b_1$ (VLA-2) integrin is a collagen receptor (33, 53, 55), whereas on many other cell types it is a receptor for both collagen and laminin (15, 31, 35). The mechanism for this cell-specific variation is unknown, but because different VLA-2 ligand-binding specificities could be retained even in solution (31), it has been suggested that functional differences may be due to post-translational modifications, or even due to variations in the primary sequences of the $\alpha$ or $\beta$ subunits. Cell type specific differences in VLA-3 functions have also been observed (18).

To learn more about the mechanism for cell type specific regulation of VLA-2 function, we have expressed a single $\alpha^2$ cDNA construct in two different cell types, to yield...
VLA-2 with three different stable but distinct patterns of functional activity. Also, we made the surprising finding that these stable functional forms could be rapidly interconverted in the presence of appropriate stimulatory mAb, but not by the phorbol ester PMA.

Materials and Methods

Antibodies and other Proteins and Reagents

Monoclonal antibodies utilized were: anti-VLA-2, 12Fl (47) and 5E6 (48); anti-VLA-5, PID6 (60); anti-VLA-β1, A-LeA5 (24), TS2/36 (22), and 4B4 (40); and the negative control antibodies P3 (37) and J-2A2 (23). Polyclonal antibody R812 directed against the COOH-terminal 22 amino acids of the integrin β1 subunit (3, 56) was obtained from Dr. A. F. Horwitz (University of Illinois, Urbana, IL). Bovine type I collagen and Matrigel were from Collaborative Research Co. (Waltham, MA); human type I collagen was obtained from Telios Co. (La Jolla, CA); and mouse laminin was obtained from Dr. Hynda Kleinman, National Institute of Dental Research, National Institutes of Health (Bethesda, MD). Collagen-Sepharose and laminin-Sepharose (each at ~0.5 mg/ml packed beads) were prepared by coupling bovine type I collagen or mouse laminin to CNBr-activated Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) according to the manufacturer's instructions.

Cell Lines

Full-length cDNA for the VLA α2 subunit (54) in the mammalian expression vector pFneo (9, 44, 49) was used for stable transfection of K562 (16) and RD (9) cells as previously described. As previously determined, background integrin levels did not change upon α2 expression. K562 cells continued to express α2, but not α1, α3, α4, or α6 (16), and RD and RDA2 cells both expressed equivalent moderate levels of α2 and α1, trace levels of α6 and α4 and no detectable α5 (9). Transfected cells were maintained in RPMI 1640 supplemented with 10% FBS, 1-glutamine and antibiotics in the continuous presence of geneticin (G-418 sulfate, Gibco Laboratories, Grand Island, NY) at 1 mg/ml.

Flow Cytometry and Matrix Adhesion Assay

Indirect immunofluorescence staining was performed using mAb against VLA protein subunits and then flow cytometric analyses were performed using a FACScan machine as described previously (16). Cell attachment to matrix proteins collagen, laminin and fibronectin was carried out in the presence or absence of stimulating or blocking antibodies as described previously (7). Briefly, cells were labeled by incubation with the fluorescein isothiocyanate (FITC)-labeled antibodies in 0.1% BSA. After 25-30 min incubation, unbound cells were removed (2-3 × 10^5 cells) by aspiration. The remaining adherent cells were fixed and permeabilized with methanol and then incubated with FITC-labeled antibodies for 1 h. After this, the cells were washed with PBS and analyzed by flow cytometry using a FACScan machine (Becton Dickinson, Sunnyvale, CA).

Using Collagen-Sepharose

Cells were surface labeled with ^125I using lactoperoxidase and lysed in the presence of 0.5% Nonidet P-40 (NP-40). Then immunoprecipitation and SDS-PAGE analyses (on 6% polyacrylamide gels) were carried out as previously described (21). For collagen-Sepharose experiments, ^125I-labeled cells were solubilized in 0.1 M Oethyl-β-D-thioglycospyranoside (OSTPG, Calbiochem Corp., La Jolla, CA), 0.1 M O-n-Octyl-β-D-glucopyranoside (OGP, Sigma Chemical Co., St. Louis, MO) 0.1 mM MnCl₂ and protease inhibitors PMSF, leupeptin, and aprotinin in PBS for 1 h at 4°C. Collagen-Sepharose and laminin-Sepharose (each at ~0.5 mg/ml packed beads) were prepared by coupling bovine type I collagen (Telios Pharmaceuticals, Inc., San Diego, CA) or mouse laminin to CNBr-activated Sepharose (Pharmacia LKB Biotechnology) according to the manufacturer's instructions.

Results

From previous experiments, it was clear that transfection of α2 into the rhabdomyosarcoma cell line RD resulted in expression of VLA-2 (here called Form-CL) that bound to both collagen and laminin (8, 9). To determine whether other α2 recipient cells would also express VLA-2 with Form-CL activity, α2 cDNA was transfected into the erythroleukemic cell line K562. After transfection, selection and enrichment, homogeneous populations stably expressing VLA-2 were obtained. Although they displayed moderate to high levels of cell surface VLA-2 (see Fig. 3 below), these cells (named KA2) consistently showed minimal adhesion to either collagen or laminin, comparable to that of mock-transfected K562 (KpF) cells (Fig. 1 A). Attempts were made to increase ligand binding activity by adding phorbol esters (50 nM, for 15 min before assay), but only marginal improvements were observed (not shown). Also, attachment assays were carried out in the presence of 1 mM Mg⁺⁺, or 0.1-1 mM Mn⁺⁺, but still no consistent increase in adhesion was observed (not shown). To indicate its lack of functional activity, the VLA-2 molecule on these cells is called “Form-O”.

To attempt enrichment for α2-transfected K562 cells that might display VLA-2 functional activity, a Matrigel selection procedure was carried out. After 4 wk of culture on Matrigel, the vast majority of cells failed to adhere and divide (Fig. 2 A). However, a few rare cells adhered to the Matrigel and then those cells proliferated to form large adherent cell colonies (Fig. 2 B). After expansion of these selected Matrigel-adherent colonies using normal tissue culture conditions (i.e., in the absence of Matrigel), they were tested for VLA-2-mediated adhesion functions. In comparison to the unselected KA2 cells, two Matrigel-selected cell cultures (KA2-M1 and KA2-M2, originating from distinct colonies) showed over 15-fold greater cell attachment to collagen, although they still bound minimally to laminin (Fig. 1 B). Notably, this enhanced binding to collagen persisted even after several additional months of continuous tissue culture.

As shown (Fig. 1 C), phorbol ester treatment of KA2-M1 and KA2-M2 cells caused a two to threefold increase in adhesion to collagen, but adhesion to laminin still remained near the background levels observed in mock-transfected K562 cells (KpF). Thus, in contrast to VLA-2 expressed in α2-transfected RD cells, which mediates adhesion to both collagen and laminin (Fig. 1 D and references 8 and 9), VLA-2 expressed in K562 cells displayed only collagen-binding activity. Because VLA-2 on the Matrigel-selected cells (KA2-M1, -M2) bound to only collagen, it was described as having "Form-C" in contrast to VLA-2 on RDA2 cells which had Form-CL activity.

Analysis of VLA-2 expression by flow cytometry (Fig. 3) showed that KA2 cells (Form-O) and KA2-M2 cells (Form-C) expressed comparable amounts of cell surface VLA-2 (detected by mAb 12Fl), whereas no VLA-2 was detected on mock-transfected (KpF) cells. Three other anti-VLA-2 mAbs gave almost identical results (not shown). Levels of the β1 subunit were increased on KA2 and KA2-M2 cells compared to KpF, consistent with there being an increase in the total amount of α subunit at the cell surface. Levels of VLA-5 were not appreciably different between KpF, KA2, and KA2-
multiple functional forms of VLA-2 derived from the same \( \alpha^2 \) cDNA. (A) Cell attachment to collagen and laminin was tested for mock-transfected K562 (KpF) and \( \alpha^2 \)-transfected K562 cells (KA2) cells. (B and C) After selection for attachment and growth on Matrigel (see Fig. 2 legend), two independently isolated subclones of KA2 cells (KA2-M1 and KA2-M2) were tested for adhesion after a 15 min pretreatment with 50 nM PMA (C), or without PMA treatment (B). (D) Unselected \( \alpha^2 \)-transfected (RDA2) cells were also tested for adhesion to collagen and laminin. Plastic surfaces in 96-well plates were coated with laminin at 10 \( \mu \)g/ml (A), 2.5 \( \mu \)g/ml (B and C), or 3 \( \mu \)g/ml (D); or collagen at 2 \( \mu \)g/ml (A), 2.5 \( \mu \)g/ml (B and C), or 1 \( \mu \)g/ml (D). Multiple other doses of collagen and laminin were tested and yielded the same relative patterns of adhesion (not shown). Doses of collagen and laminin used for D are sufficiently low so that background adhesive contributions of VLA-1 and VLA-6 are minimal.

M2 cells, and the \( \alpha^1 \), \( \alpha^3 \), \( \alpha^4 \), and \( \alpha^6 \) subunits remained essentially undetectable on these cells. Levels of VLA-2 shown here are comparable to those previously shown on \( \alpha^2 \)-transfected RDA2 (form CL) cells (8 and 9). Thus, the functional differences between VLA-2 Form-O, -C and -CL seen in Fig. 1 can not be explained on the basis of variable expression levels.

To further compare Form-O, Form-C, and Form-CL, the VLA-2 heterodimer was immunoprecipitated from KA2, KA2-M2, and RDA2 cells (Fig. 4). As shown, the anti-\( \alpha^2 \) antibody 12F1 recognized \( \alpha^2 \beta^1 \) heterodimers from KA2 (lane b), KA2-M2 (lane c), and RDA2 (lane e) cells that were essentially indistinguishable, whereas no VLA-2 subunits were seen in mock transfected cells (lanes a and d).

The human \( \beta^1 \) subunit may exist in alternatively spliced forms in which the COOH-terminal 22 amino acids of \( \beta^1 \) are replaced by either 12 (2) or 48 (36) amino acids from different exons (2). To determine whether different alternatively spliced forms of \( \beta^1 \) might correlate with different functional forms of VLA-2, we carried out immunoprecipitations utilizing rabbit antiserum specifically recognizing the COOH-terminal 22 amino acids of "nonalternatively spliced" \( \beta^1 \). The result (not shown) was that most, if not all of the \( \beta^1 \) subunit was in the nonalternatively spliced form in the K562 cells, RD cells, and other cells with Form-C or Form-CL ac-
Recent results have shown that certain anti-\( \beta \)i antibodies can cause a dramatic increase in VLA protein function (4, 32, 41, 58), possibly by causing a favorable conformational change. Here we investigated the effect of one such antibody (TS2/16) on VLA-2 function in K562 cells. As shown (Fig. 5 A) the MAb TS2/16 caused a substantial increase in collagen-mediated adhesion by both KA2 (63-fold) and KA2-M2 cells (12-fold) compared to cells stimulated with a control MAb (P3). For laminin binding (Fig. 5 B), even greater relative increases were seen (>500-fold for KA2; 70-fold for KA2-M2 cells). Thus, the TS2/16 MAb appeared to convert both Form-O and Form-C activity of VLA-2 in K562 cells into Form-CL activity, comparable to that seen in RDA2 and other cells. Another anti-\( \beta \)i antibody (A-I\( \alpha \)5) had a similar stimulatory effect on KA2 and KA2-M2 cells (not shown), and both TS2/16 and A-I\( \alpha \)5 could stimulate the function of VLA-5 endogenously expressed in K562 cells (not shown). When TS2/16 was added to RDA2 cells, adhesion to both collagen and laminin was again increased, but only by \(~\text{twofold} \) (not shown).

In comparison to TS2/16, the phorbol ester PMA yielded minimal enhancement of VLA-2 adhesion to either collagen or laminin (Fig. 6 A or B), although a stimulatory effect was more obvious when PMA was added together with TS2/16. Importantly, the MAb TS2/16 and PMA-stimulated activity was completely inhibited by the anti-VLA-2 MAb 5E8 (Fig. 5 A and B) or by the anti-\( \beta \)i antibody 4B4, thus confirming that the enhanced adhesion was indeed mediated through VLA-2. When added to RDA2 cells, PMA had almost no detectable effect (not shown).

To compare PMA and TS2/16 effects in more detail, both agents were titered, alone or together on KA2 cells, up to a maximally effective dose (Fig. 6). As shown, for adhesion to either collagen (Fig. 6 A) or laminin (Fig. 6 B), PMA was not nearly as stimulatory as TS2/16. For adhesion to collagen, effects of PMA and TS2/16 were roughly additive, whereas for adhesion to laminin, PMA appeared to act synergistically with TS2/16. In the latter case, PMA had minimal effect alone, but induced a substantial increase in adhesion when TS2/16 was also present. These results provide good further evidence for differences in the mechanisms for VLA-2 mediated binding to collagen compared to laminin.

To determine whether TS2/16 stimulation required a cellular context, or could act on soluble receptors, collagen-Sepharose binding experiments were carried out. Lysates from KA2 cells were incubated with either TS2/16 or the control mAb J-2A2, and then passed over identical collagen-Sepharose columns. Analysis of VLA-2 in both the unbound flowthrough material and material eluted with EDTA was carried out by immunoprecipitation using the anti-\( \alpha \)i mAb.

**Figure 5.** Upregulation of VLA-2 functional activity induced by MAb TS2/16. As indicated, KpF, KA2, or KA2-M2 cells were allowed to adhere to collagen (A) or laminin (B) in the presence of negative control antibody, the stimulatory antibody TS2/16, or TS2/16 plus the anti-\( \alpha \)i inhibitory antibody 5E8.

**Figure 6.** Effects of titrated MAb TS2/16 and PMA on VLA-2 function in K562 cells. Increasing doses of control antibody, TS2/16, PMA, or TS2/16 plus PMA were added to KA2 cells as indicated, and then after 15 min incubation, adhesion to collagen or laminin was tested.
Upregulation of the function of soluble VLA-2 by MAb TS2/16. (A) 125I-radiolabeled cell lysate from KA2 cells (~5 x 10^6 cell equivalents) was incubated with MAb TS2/16 (lanes a, b, d) or with control MAb J-2A2 (lanes a, c), and then added to collagen-Sepharose columns (2 ml of packed beads). After 4 h incubation at room temperature, unbound flow through material (FT) was removed by washing columns with ~10 ml of 25 mM OGP, 25 mM NaCl, 0.5 mM EDTA (wash buffer), and then bound VLA-2 was eluted by adding wash buffer containing 20 mM EDTA. Fractions (0.5 ml) containing peak radioactivity were collected and then the MAb 12F1 was used to immunoprecipitate VLA-2 from unbound (lanes a, b) and from EDTA-eluted fractions (lanes c, d). (B) In a separate experiment, KpF, KA2, and KA2-MT2 cell lysates were immunodepleted of background proteins by incubation with MAb J-2A2, followed by protein A-Sepharose. Then either J-2A2 (lanes e-g) or TS2/16 (lanes h-j) was added, and lysates were incubated batchwise with 0.1 ml of collagen-Sepharose beads (18 h, at 4°C). After washing with ~8 ml of wash buffer, specifically bound proteins were eluted using SDS-sample buffer and analyzed by SDS-PAGE as indicated.

Discussion

In highly controlled experiments, using a single α2 cDNA construct, we have been able to express VLA-2 in multiple functional forms (Forms-O, -C, -CL). In each case, the α2 subunit was expressed at similar levels and the α2 and β1 subunits showed no detectable differences in biochemical structure or in epitopes detected by various antibodies. Remarkably, Forms-O and -C could be rapidly converted to Form-CL upon addition of the anti-β1 MAb TS2/16. Together, these findings suggest that the observed variations in VLA-2 functions are not caused by differences in the primary sequence of α2, by post-translational modifications of VLA-2 subunits, or by some other inherent deficiency in the structure of the molecules. Instead, it appears that VLA-2 in different cellular environments can assume different stable, but interconvertible conformations corresponding to different functional activities.

Not only was each form consistently maintained for many months in cultured cells, but also the corresponding functional phenotype was retained when VLA-2 was solubilized. In this regard, previous studies have also shown that VLA-2 could retain the equivalent of either Form-C or Form-CL activity (depending on cell source) when solubilized, purified, and then tested in column binding assays (31) or reconstituted into liposomes (53). The challenge now remains to understand how different functional forms might be stably produced, maintained in the absence of intact cells, and yet be susceptible to conversion to a more active state by an anti-β1 MAb.

Several factors have previously been suggested to modulate the activation state of integrins and/or their ligand specificity. For example, the phospholipid environment was reported to influence the ligand specificity of the integrin αmβ2 (11), an unidentified lipid appeared to modulate the function of α1β2 (25), and α5β1 appeared to function in concert with chondroitin sulfate proteoglycan (28). For the VLA-2 results observed here, modulation by lipid factors or proteoglycans seems unlikely because ligand specificity is retained for detergent solubilized receptors. Also, divalent cations have been found to markedly influence the ligand specificity of several integrins (17, 30, 34), including VLA-2 (19, 53). However, in our studies up to 1 mM of the cations (Mn++, Mg++) known to facilitate VLA-2 function did not convert VLA-2 Form-O or Form-C into more active forms. The α chain cytoplasmic domain appears to play a key role...
in determining the functional state of α\textsubscript{m/β}_3 when expressed in CHO cells because deletion or exchange of that domain led to increased ligand binding (43). However, we have found that deletion of α\textsuperscript{c} cytoplasmic tails did not alter Form-CL activity when expressed in RD cells (8), and did not alter Form-O or Form-C activity when expressed in K562 cells (not shown). In another study, variations in glycosylation have been suggested to possibly regulate the function of VLA-5 (α\textsubscript{v}β\textsubscript{5}) (4). However, considering that (a) no obvious variation in the size of VLA-2 subunits was observed, (b) treatment with a variety of inhibitors of N-linked carbohydrate processing had no consistent effect on VLA-2 function (not shown) and (c) VLA-2 functions could be rapidly altered by adding the TS2/16 MAbs, it does not seem highly likely that glycosylation differences would account for the observed variations in VLA-2 function.

By a process termed "inside out" signaling, many stimuli, acting through a variety of receptors, can cause a rapid up-regulation of the function of β\textsubscript{1} (10, 12, 51, 61), β\textsubscript{4} (14, 45, 59, 62), and β\textsubscript{α} (5, 38) integrins without a change in integrin expression. Because phorbol esters such as PMA can mimic the integrin-activating effect of many diverse stimuli (10, 12, 45, 59, 61, 62), PKC has been invoked as having a critical role in integrin activation. In the results reported in this paper, TS2/16 had a dramatic stimulatory effect, that could not be achieved by adding PMA. Thus, because TS2/16 could act in solution, and could not be mimicked by PMA, it perhaps suggests that there may be an essential component of the activation process that does not require PKC, and cannot be overcome by adding PMA. Also, in this regard, we have found that deletion of the cytoplasmic domain of α\textsuperscript{c} abolishes PMA stimulation but not TS2/16 stimulation of VLA-4-mediated adhesion (P. Kassner, M. Hemler, manuscript in preparation). It is intriguing to speculate that TS2/16 could mimic an unknown extracellular factor that may activate integrin function independent of mechanisms involving intracellular metabolism. Consistent with this idea, peptides containing integrin recognition sequences were found to directly activate the integrin α\textsubscript{m/β}_3 (13).

The finding by others that Fab fragments of TS2/16 could stimulate the function of VLA-4, VLA-5 and VLA-6 (4, 58) indicates that receptor cross-linking is not critical, but instead favors a mechanism involving a direct induction of a conformational change by the antibody. Supporting this, the function of solubilized α\textsubscript{m/β}_3 has also been found to be directly stimulated by an anti-β subunit antibody (42). Our results argue against the suggestion by others that stimulation by anti-β MAbs requires an intact cytoskeleton and an active cellular metabolism (4, 32, 58). Most likely, inhibitors of energy dependent functions (Na\textsubscript{+}K and 2-deoxyglucose) as well as cytoskeletal disruptors (cytochalasin B) were effective in abolishing post-ligand binding events needed for cell adhesion rather than influencing conformation changes in the receptor itself induced by β\textsubscript{α} MAbs.

While we observed that partly activated VLA-2 could bind to collagen but not laminin, it is notable that increased VLA-2 binding to laminin was observed in parallel with elevated collagen binding, and in fact, we have never observed VLA-2-mediated adhesion to laminin without adhesion to collagen being at least as high. These findings with VLA-2 are perhaps reminiscent of those for α\textsubscript{m/β}_3, which in its poorly active state can only recognize immobilized fibrinogen but in a more active state can recognize soluble fibrinogen, fibronectin and other ligands (46). Similarly, partly active VLA-4 can mediate adhesion to VCAM-1 but not to the CS1 region of fibronectin, whereas fully active VLA-4 can adhere well to both ligands (39). Thus, it may be a common theme among integrins that expanded ligand specificity can occur in parallel with increased activation; a mechanism whereby additional flexibility and versatility can be achieved through only a single type of receptor. Future studies will hopefully shed more light on the reasons why it might be advantageous to have a higher threshold for activation of VLA-2 mediated adhesion to laminin, as compared to the more readily obtainable adhesion to collagen.

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