Extracellular Matrix Receptors, ECMRII and ECMRI, for Collagen and Fibronectin Correspond to VLA-2 and VLA-3 in the VLA Family of Heterodimers

Yoshikazu Takada, Elizabeth A. Wayner, William G. Carter, and Martin E. Hemler

Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 (Y.T., M.E.H.); Department of Pathobiology, University of Washington, (W.G.C.), and Department of Biochemical Oncology, Fred Hutchinson Cancer Research Center, (E.A.W.), Seattle, Washington 98104

The Very Late Activation Antigen (VLA) proteins are a family of five related heterodimers, which also are part of the integrin superfamily of cell adhesion molecules. Except for the identification of VLA-5 as a fibronectin receptor structure, the functions of the VLA proteins have remained unclarified. In this paper, immunoprecipitation experiments with both anti-α and anti-β subunit antibodies showed that the previously identified cell adhesion receptor for collagen, extracellular matrix receptor II (ECMRII), is equivalent to VLA-2. At the same time a previously described multispecific cell adhesion receptor for collagen, fibronectin, and laminin (ECMRI) has been shown to be identical to VLA-3. Although the mAb 12F1 and P1H5 both recognized VLA-2 (ECMRII), they appeared to define distinct epitopes on the α^2 subunit. On the other hand, the mAb P1B5 and J143 recognized the α^3 subunit of VLA-3 (ECMRI) at or near the same site. Consistent with the collagen receptor functions of VLA-2 (ECMRII) and VLA-3 (ECMRI), anti-VLA β antiserum blocked cell attachment to collagen.

Key words: cell adhesion, arg-gly-asp amino acid sequence, VLA proteins, integrin superfamily

A variety of cell surface structures with subunit sizes of 90,000–200,000 M_r have been described which are involved in various cell–matrix and cell–cell adhesion functions. On human cells, at least ten of these complexes have been shown to be heterodimers, belonging to the integrin superfamily of cell adhesion molecules [1]. The proteins are subdivided into three subfamilies known as 1) VLA proteins [2], 2) cytoadhesins [3], and 3) LFA-1, Mac-1, p150,95 proteins [4]. The three families all contain multiple heterodimers, and each heterodimer is composed of a common β subunit (conserved within the family) associated with a unique α subunit. Although not immunochemically

cross-reactive, there is substantial sequence homology among the different α subunits [5–10] as well as among the different β subunits [6,11–13]. Also there is functional homology, since many of the integrin receptors may recognize ligands which contain arg-gly-asp or a closely related amino acid sequence. [14].

Within the integrin superfamily, a family of five heterodimers called VLA proteins has been defined [2]. The "very late antigens" VLA-1 and VLA-2 were originally found on activated T lymphocytes [15–18] and subsequently, analyses of other cell types lead to the discovery of three additional heterodimers (VLA-3, VLA-4, VLA-5), each with distinct α subunits, and sharing the same β subunit [2]. The VLA-5 structure which is present on the myeloid cell lines K-562 and U-937 is immunochemically identical [19] to a fibronectin receptor (FNR) structure affinity purified from human placenta [20] and osteosarcoma cells [21]. In confirmation of this identity, the N-terminal sequence of the VLA-5 α^5 subunit [5] was essentially the same as that obtained from FNR α subunit cDNA sequence [6,10]. Functions of the other VLA heterodimers have not been conclusively demonstrated. However, the VLA-3 heterodimer has been shown to be immunochemically cross-reactive with the avian CSAT structure [19], which has both fibronectin and laminin receptor activity [22,23].

Identification of the VLA proteins (and other integrin structures) and understanding of their $\alpha\beta$ subunit organization now provides a framework for analyzing and comparing other newly discovered structures. In this regard, structurally and functionally related glycoproteins that mediate fibroblast adhesion to specific components of the extracellular matrix (ECM) were described and designated ECMRI and ECMRII [24]. Monoclonal antibody (mAb) P1H5 (which sees ECMRII) specifically inhibited cell adhesion to collagen, while monoclonal antibody P1B5 (recognizing the promiscuous ECMRI) inhibited fibroblast adhesion to fibronectin, laminin, and collagen [24]. Both members of this ECMR family contained a common β subunit and a unique α subunit, each of approximately 140,00 M_r. The epitopes recognized by the anti-ECMRI and anti-ECMRII mAb were shown to be present on the α subunits, indicating that the α subunits determined ligand binding specificity. Because the size and $\alpha\beta$ subunit organization of the ECMRI and ECMRII complexes resembled the VLA proteins, studies were carried out to directly compare these structures and thus determine their potential relatedness.

MATERIALS AND METHODS
Antisera and Monoclonal Antibodies

The mAb TS2/7 [16], 12F1 [25], J143 [26], and B-5G10 [27] recognize the α^1, α^2, α^3, and α^4 subunits of VLA-1, VLA-2, VLA-3, and VLA-4 respectively [2]. Although a mAb specific for VLA-5 has not yet been described, that structure has been defined and purified from K-562 cells (which do not contain any other VLA heterodimers [2]) or from placenta (after all of the other VLA proteins have been removed [5]). The mAb A-1A5 specifically recognizes the β subunit common to all VLA proteins [2,15,18]. The mAb P1H5 and P1B5 were prepared as described [24] and were found to inhibit cell adhesion to collagen alone, or to collagen, fibronectin, and laminin, respectively. Rabbit anti-native VLA β subunit and rabbit anti-denatured VLA β subunit were prepared as previously described [5]. Specificity of rabbit serum for the VLA β subunit was confirmed by immunoblotting. Purified IgG was obtained from rabbit heteroantisera by using a protein A-Sepharose column as described [28] and was dialyzed into PBS for use in inhibition studies.

Antigen Purification and Immunoprecipitation

Purified VLA-1, VLA-2, VLA-3, VLA-4, and VLA-5 proteins were prepared from extracts of placenta, platelets, and T lymphoblastoid cell lines by using mAb-Sepharose columns as previously described [5]. Immunoprecipitation was carried out using either ^{125}I-labeled purified VLA antigen or ^{125}I-labeled whole cell extracts, as previously described [2,27]. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis on 7% acrylamide gels.

Cell Adhesion Assay

Collagen (approximately 1 μg in 100 μl dH$_2$0) was coated onto 96-well flat-bottom Linbro microtiter plates by evaporation to dryness. Type I collagen (prepared from rat tails) was a gift from Dr. S. Sugrue (Harvard Medical School), and type IV collagen was obtained from Collaborative Research Co. Collagen-coated wells were then incubated with BSA (10 mg/ml) for 30 min at 37°C and washed 3 times with PBS. Then 50,000-75,000 cells were added per well. Following trypsinization and recovery in the presence of 10% fetal calf serum, cells from the neuroblastoma line SK-N-SH were washed in serum-free alpha or MEM medium, and 50,000 cells in 100 μl of medium were added per well. After 2 hr at 37°C, media and unattached cells were shaken out of the plate, and 1% crystal violet in methanol was added to each well. After 2–3 min, the plate was washed in distilled H$_2$O and allowed to dry, and then the OD$_{550}$ for each well was determined by using a 96-well plate reader.

RESULTS

Comparison by Immunoprecipitation

A cell-surface glycoprotein complex (ECMRII) of approximately 147,000/125,000 M$_r$, recognized by the mAb P1H5, has recently been implicated in fibroblast adhesion to collagen [24]. At the same time, a related structure (ECMRI) of 145,000/125,000 M$_r$ (nonreducing), recognized by the mAb P1B5, has been implicated in fibronectin, laminin, and collagen binding [24]. Because these structures have sizes potentially similar to VLA proteins, the ability of the mAb P1H5 and P1B5 to react with VLA proteins was tested. As shown in Fig. 1, P1H5 directly immunoprecipitated immunopurified VLA-2 but did not recognize VLA-3. Conversely, P1B5 precipitated VLA-3 but did not recognize VLA-2. Neither VLA-2 nor VLA-3 was seen by the negative control mAb (lanes a,b), and neither P1H5 nor P1B5 recognized purified VLA-1, VLA-4, OR VLA-5 (not shown). Purified VLA-2 and VLA-3 (without immunoprecipitation) are shown in lanes g,h. These results suggested that P1H5 recognized the α^2 subunit of VLA-2, whereas P1B5 recognized the α^3 subunit of VLA-3. If either antibody had recognized the common VLA β subunit, it would have precipitated multiple VLA heterodimers. To confirm that the structures (recognized by P1B5 and P1H5) shared the common VLA β subunit and are wholly contained within the subset of VLA protein heterodimers, a preclearing experiment was carried out (Fig. 2). Removal of all VLA proteins by using the anti-VLA β mAb A-1A5 completely removed all reactivity with 12F1, J143, P1H5, or P1B5. Thus the common β subunit previously shown to be shared by the P1H5 and P1B5 antigens (ECMRII and ECMRI, respectively) [24] is the same as the common VLA β subunit.
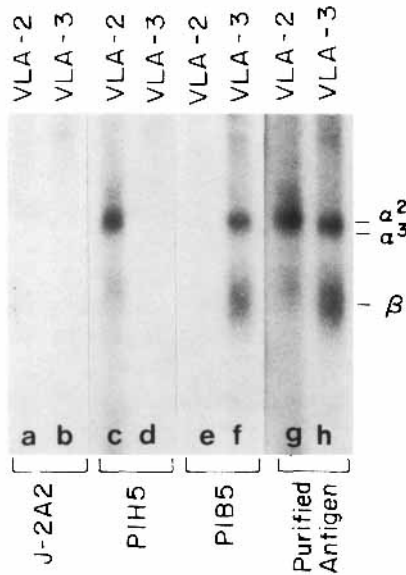
Fig. 1. Immunoprecipitation of purified VLA proteins by the mAb P1B5 and P1H5. ^{125}I-radio-labeled aliquots of immunoaffinity-purified VLA-2 and VLA-3 were tested for immunoprecipition by using the negative control mAb J-2A2 (**lanes a,b**), the mAb P1H5 (**lanes c,d**), the mAb P1B5 (**lanes e,f**), or were analyzed directly (without immunoprecipitation; **g,h**) by SDS-PAGE on a 7% acrylamide gel.
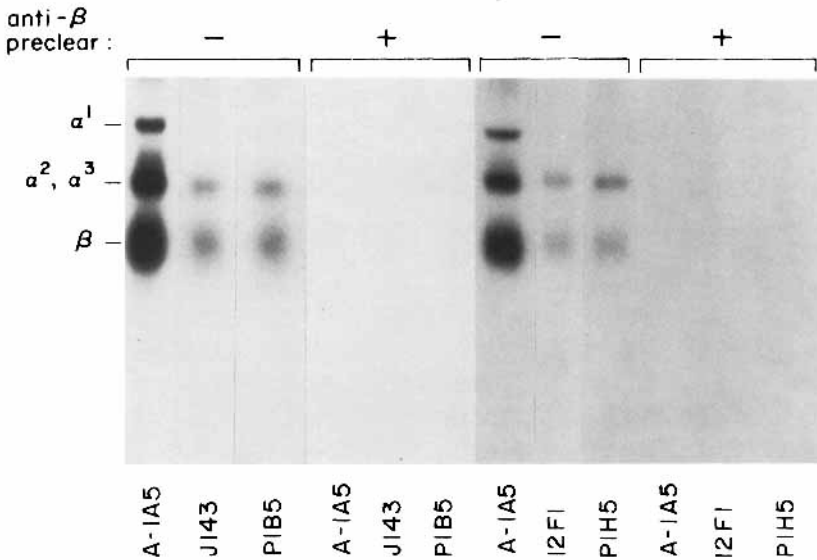

Fig. 2. Preclearing of P1B5 and P1H5 antigens by the anti-VLA β mAb A-1A5. ^{125}I-radiolabeled extracts from the cell line MG-63 were precleared with a negative control antibody (−) or with the anti-VLA β antibody A-1A5 (+). Then the remaining extract was immunoprecipitated by using A-1A5, J143 (anti-α^3), P1B5, 12F1 (anti $-\alpha^2$), or P1H5 as indicated.

Comparisons by Competitive Binding

To further analyze P1H5 and P1B5 reactivity with VLA proteins, epitope mapping studies were carried out. As shown in Fig. 3A, P1B5 readily blocked binding of radiolabeled J143 antibody to MG-63 cells, almost efficiently as J143 blocked its own binding. Thus J143 and P1B5 may recognize similar or overlapping epitopes on the α subunit of VLA-3. The anti-VLA-2 antibodies 12F1 and P1H5 did not block ^{125}I-J143 binding.

In a separate experiment (Fig. 3B), binding of ^{125}I-12F1 to platelets was inhibited by unlabeled 12F1, but not by P1H5. This indicates that 12F1 and P1H5 recognize distinct epitopes on the VLA α^2 subunit.

Inhibition of Cell Attachment

Attachment of SK-N-SH cells to Type I or Type IV collagen could not be inhibited by the 12F1 mAb (up to 1 mg/ml), thus suggesting that the 12F1 epitope is structurally, as well as functionally, distinct from the P1H5 collagen-binding epitope. However, rabbit anti-VLA β serum did block the attachment of SK-N-SH cells to collagen, as seen by the decrease in the number of crystal violet stained cells (Fig. 4). In a more quantitative experiment, anti-VLA β serum blocked attachment of SK-N-SH cells to type I or type IV collagen by 85% (Fig. 5). Control sera, including normal rabbit sera, or antisera directed against denatured VLA β subunit, failed to block attachment. Experiments with purified anti-VLA β antibodies (0.5 and 1.0 mg/ml) yielded essentially the same results, blocking cell attachment by 60%–80%, whereas antibodies from normal rabbit serum did not. Other cell lines, including the osteosarcoma cell line MG-63 were also inhibited from attaching to collagen (70%–80%) by the same serum (not shown). The blocking effect seen in Figures 4 and 5 was somewhat specific since attachment to vitronectin or nonspecific attachment to tissue culture plastic was not blocked.

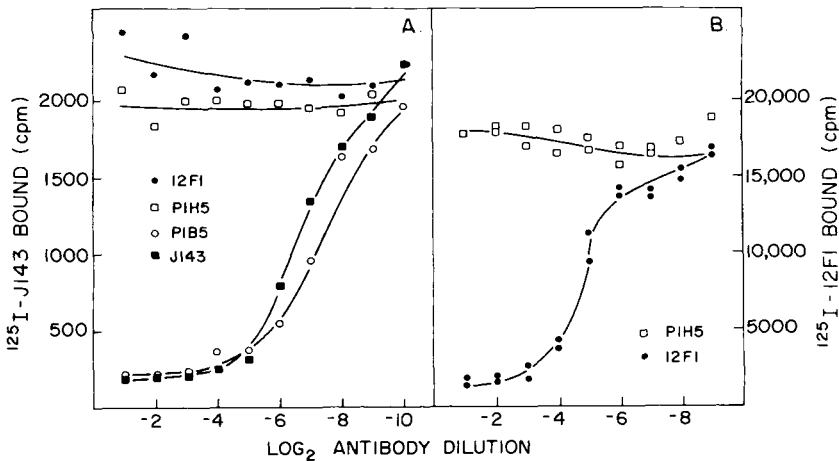Although J143 and P1B5 appear to bind to related epitopes, J143 failed to inhibit

Fig. 3. Comparison of epitopes recognized by P1B5, P1H5, and anti-VLA mAb. **A.** Binding of ^{125}I-trace-labeled J143 (200,000 cpm/well) to MG-63 cells (300,000/well) was carried out in the presence of unlabeled J143, 12F1, P1H5, and P1B5 as indicated. Serial two-fold dilutions of inhibitor mAb were present, starting with 2–5 µg of unlabeled mAb per well. **B.** Binding of ^{125}I-trace-labeled 12F1 to platelets was carried out in the presence of serial dilutions of unlabeled 12F1, or P1H5, as indicated.
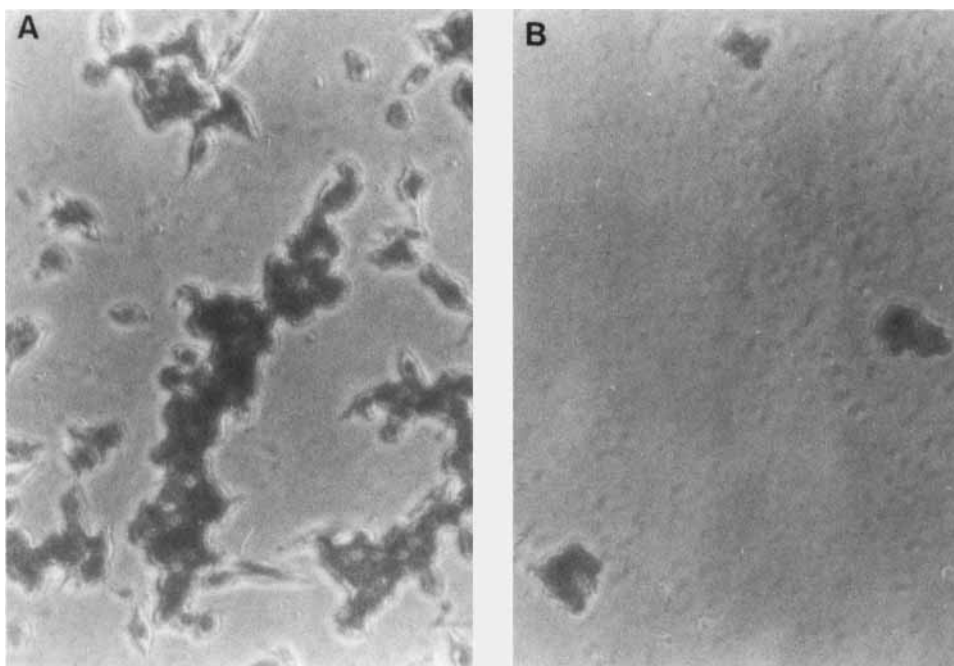
Fig. 4. Inhibition of cell attachment to collagen by anti-VLA β serum. Attachment of SK-N-SH cells to plastic coated with Type I collagen was assessed in the presence of anti-VLA β heteroantiserum (**B**) or control antiserum (**A**) as described in Materials and Methods.

cell attachment to fibronectin or laminin, and thus does not resemble P1B5 [24] in that respect.

DISCUSSION

Collagen Receptor Function for VLA-2

The results in this paper clearly establish that VLA-2 is identical to ECMRII, a structure which has collagen receptor activity and was previously defined using the mAb P1H5 [24]. The mAb P1H5 was previously shown to recognize ECMRII but not ECMRI [24] and is reported here to specifically recognize purified VLA-2 and not any other VLA heterodimer. Both results are consistent with recognition of an epitope on the α^2 subunit of VLA-2, rather than on the β subunit common to all VLA proteins. Because anti-VLA-2 mAb 12F1 and P1H5 did not compete in binding studies and because P1H5 [24], but not 12F1, blocked cell adhesion to collagen, it suggests that separate epitopes on the α^2 subunit are recognized by the two antibodies. Like P1H5 [24], antiserum to the VLA β subunit also blocked cell attachment to collagen, suggesting that β subunit as well as the α subunit may be involved in ligand binding. However, anti-VLA β blocking results must be interpreted with caution, since another collagen receptor structure (VLA-3 or ECMRI) is present on many of the same cells which express VLA-2.

The indication in this paper that VLA-2 is a collagen receptor is consistent with the results of other studies. The α^2 and β subunits of VLA-2 were found to be identical to glycoproteins Ia and IIa in the classic platelet nomenclature [29]. In addition, it was
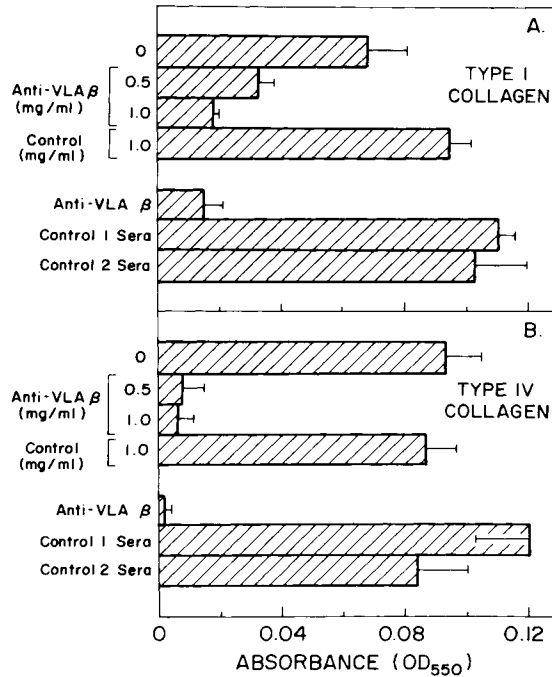
Fig. 5. Quantitation of anti-VLA β serum inhibition of cell attachment to collagen. After trypsinization, SK-N-SH cells (50,000/well) were added to wells coated with type I (upper panel, A) or type IV (lower panel, B) collagen. Included in the well was 0, 0.5, or 1.0 mg/ml of purified anti-VLA β rabbit IgG, or 1.0 mg/ml of purified control rabbit IgG. In a separate experiment, samples of whole rabbit serum (dilited 1/10) including anti-native VLA-β, normal rabbit serum (control 1), and rabbit anti-denatured VLA β (control 2) were tested for inhibitory effects. Numbers reflect absorbance of attached, stained cells in wells as measured by a 96-well plate reader.

found that human fibroblast class II extracellular matrix receptor (ECMRII) mediates platelet adhesion to collagen and is also identical to the platelet–glycoprotein Ia-IIa complex [38]. Thus, when a patient who lacked expression of platelet protein Ia (α^2) also had an abnormal deficiency in platelet responsiveness to collagen [30], a further link was provided between VLA-2 and collagen receptor function.

In other studies, proteins of 155,000/125,000 M_r [31] and 160,000 M_r [32] have been isolated from stimulated U-937 cells [31] or platelets [32] by using collagen-Sepharose affinity matrix. Although not yet directly compared, the similarity in size of these proteins to the subunits of VLA-2 (165,000/130,000 M_r) suggests that they may be the same. However, VLA-2 is probably unrelated to several other proteins associated with collagen receptor function, since they are clearly quite different in size [24,33,34].

VLA-3: An Adhesion Receptor With Multiple Ligands

Results in this paper suggest that human VLA-3 is identical to ECMRI, a promiscuous receptor for fibronectin, laminin, and collagen [24]. This result is consistent with previous results, showing that VLA-3 is immunochemically related to the band 2 and band 3 subunits [19] of a fibronectin and laminin receptor structure called CSAT [22,23]. Within the integrin superfamily, there is precedent for one receptor having

TABLE I. Summary of VLA Proteins and Associated Functions

VLA Heterodimer	Ligands	MAb	Reference
VLA-1	?	TS2/7	16,17
VLA-2 (ECMRII)	Collagen I,III,IV,V,VI	12F1,P1H5	24,25
VLA-3 (ECMRI)	Collagen I,IV Fibronectin, Laminin	J143,P1B5	24,26
VLA-4	?	B-5G10	27
VLA-5 (FNR)	Fibronectin	—	2,19

multiple ligands, since the GPIIb/IIIa complex can recognize fibronectin, fibrinogen, and von Willebrand factor [1,14].

Although VLA-3 (ECMRI) has fibronectin receptor activity, it previously has been shown to have an α subunit distinct from the fibroblast fibronectin receptor (VLA-5), while sharing a common β subunit [2,24]. Multiple fibronectin receptor structures, with unique α subunits and a common β subunit, have also been found on rat cells [35]. It is suspected that the rat structures might be VLA-3 (ECMRI) and VLA-5 analogues.

When human fibronectin receptor was affinity-purified from MG-63 cells [21], a homogeneous structure corresponding to VLA-5 [19] was obtained, despite the high level of VLA-3 expression on that cell line [27]. It may be significant that the fibronectin receptor (VLA-5) has been eluted from fibronectin affinity columns by using peptides containing arg-gly-asp sequences [20,21], whereas ECMRI (VLA-3) was eluted by using NaCl [24]. Also, VLA-3 might have a different affinity for certain fibronectin fragments used for affinity purification, or it may recognize a different portion of the fibronectin molecule. In this regard, it can be speculated that multiple fibronectin receptors might correlate with the multiple cell binding sequences known to be present in the fibronectin molecule [36,37]. Anti-VLA β serum previously was shown to block cell attachment to fironectin [19]. However, at this time, it is not clear whether that result was due to blocking the function of VLA-3 or VLA-5 or both together. An updated summary of VLA protein functions is listed in Table 1.

ACKNOWLEDGMENTS

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