Multiple Very Late Antigen (VLA) Heterodimers on Platelets

EVIDENCE FOR DISTINCT VLA-2, VLA-5 (FIBRONECTIN RECEPTOR), AND VLA-6 STRUCTURES*

(Received for publication, December 11, 1987)

Martin E. Hemler‡, Carol Crouse‡, Yoshikazu Takada‡, and Arnoud Sonnenberg‡

From the §Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 and the ¶Division of Tumor Biology, The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

After removal of very late antigen (VLA) 2 material from a radiolabeled detergent lysate of platelets, another VLA heterodimer was precipitated using antibody to the common VLA β subunit. This structure was identified as VLA-5 because it contained VLA β plus an α subunit that was (i) recognized by anti-α2 antibodies and (ii) cleaved by V8 protease to yield a characteristic α2-like pattern of peptide fragments. Besides VLA-2 and VLA-5, a third heterodimer, here named VLA-6, was also present on platelets. VLA-6 (an αβ complex) was defined using the monoclonal antibody GoH3 (Sonnenberg, A., Janssen, H., Hogervorst, F., Calafat, J., and Hilgers, J. (1987) J. Biol. Chem. 262, 10376–10383). Although it resembled VLA-5 in size, VLA-6 was different from VLA-5 because (i) removal of the αβ subunit did not remove α2, (ii) removal of α8 by the GoH3 antibody did not remove α8, (iii) the α6 and α8 subunits had very distinct one-dimensional V8 peptide maps, and (iv) the α6 and α8 subunits had distinct migration patterns on two-dimensional O'Farrell gels. The β subunit of VLA-6 was identified as the common VLA β subunit because (i) it was recognized by anti-VLA-β antibody and (ii) it yielded a V8 protease cleavage map characteristic of β. VLA-6 was not readily seen in VLA-5 immuno precipitations, apparently because the α8 subunit is only loosely or partially associated with the VLA β subunit. Because VLA-5 and VLA-6 both closely resemble the previously defined Ic-IIa platelet protein complex, it is likely that there is more than one platelet “Ic” protein complexed with IIa.

Evidences is emerging that several cell surface heterodimers in the integrin superfamily of cell adhesion molecules (1), sometimes known as I-SCAM, are present on platelet surfaces (2), thus suggesting a greater variety and complexity of platelet adhesion functions than previously thought. The I-SCAM proteins include at least 10 different cell-matrix and cell-cell adhesion receptor heterodimers, subdivided into three families known as (i) VLA proteins (3), (ii) cytoadhesins (2), and (iii) lymphocyte functional antigen-1, macrophage antigen-1, p150,95 proteins (4). All or most of these receptors have significant structural homologies, and may recognize ligands which contain Arg-Gly-Asp or a closely related amino acid sequence (5).

Perhaps the most studied member of I-SCAM found on platelets is the glycoprotein IIb-IIIa structure (2), which mediates platelet binding to fibronectin, fibrinogen, and von Willebrand factor. In addition, the heterodimer VLA-2 has been found on platelets (6), and the α2 and β subunits of VLA-2 have recently been shown to correspond to platelet proteins Ia and IIa (7). Because the platelet protein Ia was deficient on a patient's platelets that were unresponsive to collagen (8), the suggestion was that VLA-2 might be a collagen receptor. In support of this, an antibody that specifically was found to block cell adhesion to collagen (9), also recognized VLA-2. Furthermore, proteins of M, 155,000/125,000 (10) and M, 160,000 (11) which are similar in size to the subunits of VLA-2 (M, 165,000/130,000) have been isolated from stimulated U-937 cells (10) or platelets (11) using collagen-Sepharose affinity matrix.

At the same time that the platelet Ia/IIa heterodimer was identified as VLA-2, it became obvious that a complex of platelet proteins Ic and IIa might also be a part of the VLA family of heterodimers (7). Although it was not established which VLA heterodimer might correspond to Ic and IIa, likely candidates were VLA-3 and VLA-5, based on their characteristic reduced/nonreduced migration of subunits. Notably, both immunological analyses (12) and NH2-terminal sequence (13, 14) have indicated that VLA-5 is identical to the fibronectin receptor isolated from human placenta (15) or from an osteosarcoma cell line (16). Others have found a complex of platelet surface antigens which was cross-reactive with fibroblast fibronectin receptor (17). This would be expected since the common VLA β subunit, found in fibroblast fibronectin receptor (VLA-5 (12)) is equivalent to platelet protein IIa (7).

In another study, the mAb GoH3 was shown to recognize an epitope on platelet glycoprotein Ic (18). Also, GoH3 immunoprecipitated a complex of Ic and IIa proteins from human and mouse platelets, as well as from a variety of other cell types including epithelial cells (18). The nonreduced/reduced properties of the GoH3 antigen complex suggested that it may be related to members of I-SCAM, with most potential similarity to VLA-3 and/or VLA-5.

In this study, we have determined more precisely which VLA heterodimers besides VLA-2 are present on platelets. Also, the relationship between VLA proteins and the “Ic-IIa”

*This work was supported by National Institutes of Health Grants CA42368 and GM38903 (to M. E. H.) and Grant NKI 849 from The Netherlands Cancer Foundation (to A. S.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡To whom correspondence should be addressed.
1The abbreviations used are: I-SCAM, integrin superfamily of cell adhesion molecules; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VLA proteins, very late antigens first discovered on activated T cells, but now known to have wide cell distribution; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
§Dana-Farber Cancer Institute, Boston, MA.
¶Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands.

7660
proteins recognized by mAb GoH3 was examined. In the process, evidence for three distinct VLA heterodimers on platelets was obtained, and the existence of a novel VLA heterodimer, called VLA-6, was established.

MATERIALS AND METHODS AND RESULTS

The reduced/nonreduced properties of the material visible in Fig. 1, lanes f and i, suggested that it could be VLA-3 and/or VLA-5 (3). However, since VLA-3 is absent on platelets (lanes c and h), VLA-5 was the more likely possibility. Thus further experiments were carried out to directly look for the $\alpha^5\beta^3$ subunit of VLA-5 on platelets. Immunoblotting of platelet-derived material was carried out using antiserum prepared against affinity-purified $\alpha^5\beta^3$ complex (Fig. 2). As shown, this serum recognized both $\alpha^5$ and $\beta^3$ proteins from the positive-control K-562 whole cells (lane a), from whole platelets (lane b), and from affinity-purified platelet VLA protein (lane c). The same serum recognized only $\beta$ subunit but not $\alpha^5$ (lane d) from a purified $\alpha^5\beta^3$ preparation. Thus the anti-$\alpha^5$ antibodies in this serum (seen in Fig. 2, lanes a–c) did not recognize $\alpha^5$, just as the anti-$\alpha^5$ mAb 12F1 did not recognize $\alpha^5$ in Fig. 1. In a control experiment, the presence of $\alpha^5$ in the $\alpha^5\beta^3$ preparation

(used in Fig. 2, lane d) was confirmed by its selective recognition by anti-$\alpha^5$ rabbit serum (lane g). In additional control experiments, anti-$\beta$ serum selectively recognized $\beta$, but not $\alpha^5$ from K-562 or platelets (lanes e and f), affirming that the anti-$\alpha^5$ reactivity seen in lanes a–c is distinct from anti-$\beta$ reactivity present in the same serum preparation. Also, the anti-$\alpha^5$ serum used in lanes a–c did not cross-react with purified VLA subunits $\alpha^1$, $\alpha^5$, or $\alpha^6$ (not shown). It is not clear why $\alpha^5$ in lane a has a higher apparent $M_r$, than in lanes b and c, but variable glycosylation is suspected.

To further establish the identity of the "$\alpha^5\beta$" subunit on platelets, it was directly compared to a prototype $\alpha^5$ subunit (3) (obtained from K-562 cells) by V8 protease mapping. Following preclearing of platelet VLA-2 protein (using mAb 12F1, as in Fig. 1B), $\alpha^5$ from platelets or from K-562 cells were immunoprecipitated using A-1A5, and separated from $\beta$ subunits by nonreduced SDS-PAGE (not shown). Then gel pieces containing the $^{125}$I-labeled $\alpha^5$ proteins were excised, digested with V8 protease, and analyzed in a second SDS gel (Fig. 3). The peptide fragments of $\alpha^5$ derived from either K-562 cells (lanes a and c) or platelets (lanes b and d) appeared to be nearly identical after digestion at two different protease concentrations. This suggests that under these experimental conditions $\alpha^5$ is the only other VLA subunit besides $\alpha^5$ which is readily detectable in A-1A5 precipitations from $^{125}$I-platelet extract.

To better understand the possible relation between the GoH3 antigen(s) and VLA proteins compared in Fig. 4, a precoating experiment was carried out (Fig. 5). When all VLA $\beta$ reactivity was entirely removed (lanes d–f), associated $\alpha$ subunit material (presumably $\alpha^5$ and $\alpha^6$) was also removed (compare lanes a and b with d and e). At the same time, M, 110,000 $\beta$-like subunit (seen in lane c) was removed from the

![Fig. 2. Identification of platelet VLA subunits by immunoblotting](image)

![Fig. 3. Analysis of platelet $\alpha^5$ subunit by V8 protease mapping](image)
FIG. 5. Comparison of platelet VLA proteins with GoH3 antigens by preclearing analysis. Cell extract from \(^{125}\)I-labeled platelets was precleared using either negative control mAb (J-2A2), anti-VLA \(\beta\) antibody (mAb A-1A5 together with rabbit anti-\(\beta\)) anti-VLA-2 mAb (12F1), or the mAb 12F1 and GoH3 together, as indicated at the top of the figure. After three successive preclearing steps, the remaining extract was used for specific immunoprecipitation with A-1A5 (for the remaining VLA-\(\beta\) associated protein), 12F1 (for VLA-2), and GoH3 (for remaining GoH3 reactive antigens) as indicated at the bottom of the figure.

GoH3 precipitation (lane f), but little or none of the GoH3 \(\alpha\) subunit was removed (lane j). Thus "\(\alpha\)" subunit seen by GoH3 (hereafter called \(\alpha\)') is not tightly associated with the VLA-like \(\beta\) subunit. Preclearing with mAb 12F1 (lanes g–i) removed all VLA-2 material as expected (lane h), but did not remove VLA-5 (lane g), and did not diminish the level of \(\alpha\) as precipitated by GoH3 (lane i). Simultaneous preclearing using both mAb 12F1 and GoH3 (lanes j–l and n) again resulted in the expected removal of \(\alpha\)' (lane k), and caused over 90% removal of \(\alpha\)" (lane l), but did not noticeably diminish the level of \(\alpha\)" either as precipitated by A-1A5 (lane f) or by anti-\(\alpha\)" serum (compare lanes m and n). Because the antibodies used in these preclearing experiments (GoH3, A-1A5, 12F1, rabbit anti-\(\beta\)) recognize VLA subunits independent of glycosylation (19, 20), it is unlikely that the newly described \(\alpha\)" subunit is simply a glycosylation variant of a previously characterized subunit.

To further analyze the tentatively assigned "VLA-6" subunits (\(\alpha\)" and \(\beta\)), V8 protease mapping was carried out (Fig. 6). As shown, V8 digestion of \(\beta\) subunit from precipitations utilizing anti-VLA \(\beta\) antibody compared to mAb GoH3 (anti-VLA-6) yielded identical patterns at two different protease concentrations. In contrast, digestion of purified \(\alpha\)", \(\alpha\)" and \(\alpha\)" subunits yielded distinct patterns at two different protease concentrations. This result further supports the concept that there is a distinct VLA-6, composed of a unique \(\alpha\)" subunit associated with the common VLA \(\beta\) subunit.

Although VLA-5 and VLA-6 showed similar subunit migration patterns in one-dimensional SDS-PAGE, differences could readily be detected in two-dimensional isoelectric focusing/SDS-PAGE gels (Fig. 7). Analysis of precipitations using A-1A5 (A panels) or GoH3 (B panels) showed that \(\alpha\)" appeared to be more basic (a series of spots between pH 5.0 and 5.4) compared to any of the subunits in A-1A5 precipitated material. A second dimension slab gel SDS-PAGE was carried out nonreduced (NR), or reduced (R). The pH gradient was determined by analyzing 1-cm pieces cut from a tube gel after isoelectric focusing.
material above $\alpha ^{\epsilon}$ (at pH 5.5) could be a trace of trailing $\alpha ^{n}$ material, or possibly a separate, unknown protein. Analysis of VLA proteins from K-562 cells (D panels) showed that $\alpha ^{c}$ resembles $\alpha ^{n}$ when nonreduced, and co-migrates with $\beta$ when reduced. Thus unlike $\alpha ^{e}$, the $\alpha ^{o}$ subunit is not readily distinguishable from $\alpha ^{c}$ and $\beta$ in these gels.

A standard approach for distinguishing platelet surface proteins (e.g. Ia, Ila, Ic, Iib etc.) has involved the use of two-dimensional reduced/nonreduced gels (27–30). When A-1A5 immunoprecipitates were analyzed in two-dimensional reduced/nonreduced gels, distinct spots corresponding to $\alpha ^{e}$, $\alpha ^{o}$, and $\beta$ were observed (Fig 8, panel A). The $\alpha ^{e}$ assignment was confirmed by analysis of VLA-2 alone (panel B) and the location of $\alpha ^{o}$ was supported by analysis of VLA-5 from K-562 cells (panel C). Importantly, the position of $\alpha ^{e}$ relative to $\beta$ in a GoH3 immunoprecipitation (panel E) closely resembled the location of $\alpha ^{o}$ in panels A and C. Consistent with this, a mixed sample containing both A-1A5 and GoH3 precipitations (panel D) failed to show separate spots for $\alpha ^{e}$ and $\alpha ^{o}$, as they both appear to migrate in a position expected for platelet protein Ic.

**DISCUSSION**

The results in this paper clearly establish that the fibronectin receptor structure (VLA-5) and a new VLA heterodimer (VLA-6) are present on platelet surfaces, along with the previously described VLA-2 complex (6, 7).

**VLA-5 on Platelets**—The presence of this structure on platelets has previously eluded notice, primarily because (i) no mAb specifically recognizing VLA-5 has yet been reported, and (ii) the $\alpha ^{e}$ subunit migrates similar to $\alpha ^{n}$ when nonreduced, and migrates near $\beta$ when reduced, in both one-dimensional and two-dimensional O’Farrell gels. In this paper, removal of $\alpha ^{n}$ by preclearing has uncovered the $\alpha ^{e}$ subunit, and its identity has been confirmed by (i) recognition by $\alpha ^{e}$-specific heteroantiserum and (ii) V8 protease mapping in comparison to known $\alpha ^{c}$. Because VLA-5 is a fibronectin receptor (12), it will be interesting to analyze the relative functional contribution of this structure, compared to the other platelet fibronectin receptor glycoprotein Iib-Illa. Some functions previously attributed to glycoprotein Iib-Illa could possibly be carried out by VLA-5. Also, it is not yet known how the function of this receptor might be altered by platelet activation.

**VLA-6 on Platelets**—Although the antigens recognized by the mAb GoH3 have similar reduced/nonreduced properties to VLA-5 (e.g. see Fig. 8), this structure is clearly different in several critical respects. Unlike VLA-5, the VLA-6 structure is (i) not present on K-562 cells, (ii) does not have tightly associated $\alpha$ and $\beta$ subunits, and (iii) is variably co-precipitated together with additional proteins of $M$, 85,000 and $>300,000$. Furthermore, the $\alpha ^{e}$ and $\alpha ^{o}$ subunits can be readily distinguished by O’Farrell two-dimensional gel patterns, by one-dimensional V8 peptide maps, and by selective antibody reactivity with either anti-$\alpha ^{e}$ serum or mAb GoH3. A summary of the properties which distinguish the $\alpha ^{e}$, $\alpha ^{o}$, and $\alpha ^{n}$ subunits is shown in Table I.

It is not surprising that $\alpha ^{n}$ eluded detection in previous VLA analyses because little or no $\alpha ^{n}$ could be co-precipitated with the VLA $\beta$ subunit using our experimental conditions. As seen by V8 protease mapping, O’Farrell two-dimensional gels, and preclearing experiments, it appeared that $\alpha ^{e}$ and $\alpha ^{o}$, rather than $\alpha ^{n}$, were co-precipitated with VLA $\beta$. A possible explanation is that a weak association between $\alpha ^{n}$ and $\beta$ could be disrupted during radiolabeling and preparation of cell extract (as previously suggested, see Ref. 18) and/or could be disrupted by anti-$\beta$ antibodies. Although no association was detectable using anti-$\beta$ antibodies and only little association was seen using the anti-$\alpha ^{e}$ antibody (GoH3), the $\alpha ^{e}$ and $\beta$ subunits are known to associate from studies involving sucrose gradients and cross-linking (18). Also there is some evidence from sucrose gradient studies that $\alpha ^{e}$ could be present in excess of available $\beta$ subunit, thus further accounting for its apparent dissociated state. The apparent weak $\alpha ^{e}\beta$ association seen for VLA-6 is reminiscent of VLA-4, the only other VLA heterodimer to show weak association. Also, perhaps if analogy to VLA-4 can be further extended, the $M$, 85,000 peptide co-precipitated with VLA-6 might be a product of cleavage of $\alpha ^{e}$, just as $\alpha ^{o}$ of VLA-4 yields an $M$, 80,000 cleavage product (21).

The function of VLA-6 is unknown, but it is likely to be
another cell-matrix adhesion structure because (i) it is a member of the VLA family of heterodimers, and (ii) the GoH3 antibody stains the basolateral regions of polarized mammary epithelial cells (18).

The question of the true identity of platelet IC protein is perhaps best solved by suggesting that there are two distinct IC proteins, corresponding to both α5 and α6. The traditional reduced/nonreduced gel analysis used to assign IC cannot resolve α6 from α5 since they both migrate identically to IC (see Fig. 8). Perhaps fortunately, VLA-3 is not also present on platelets, or else there would be a third IC protein.

In summary, it is now clear that platelets have at least four adhesive capabilities are assumed. A summary of I-SCAM members and their traditional platelet names is shown in Table II. The wealth of information now being obtained in studies of these proteins on other cell types can now be applied to platelet studies.

REFERENCES
VLA-2, VLA-5, and VLA-6 on Platelets

Figure 1: Analysis of VLA/intergrin heterodimers on platelets. Part A, Total VLA (lane A). VLA-2 (lane b), VLA-5 (lane c), VLA-6 (lane d), and GPIIb-IIIa (lane e) were immunoprecipitated from 125I-labeled platelet extract using the mAb A-245, B-211, J409, B-520, and TIB respectively, and analyzed without reduction. Part B, After removing nearly all VLA-2 from a sample of platelet lysate by three successive immunoprecipitations using the antibody against VLA-2 (lanes f-j), VLA-3 (lanes k-l) and VLA-5 (lane h) by immunoprecipitation using the appropriate mAb. Samples were analyzed nonreduced (NR) or reduced (R) as indicated.

Figure 2: Comparison of platelet VLA proteins with proteins immunoprecipitated using the G011 antibody. Proteins, recognized by the mAb G011 (lanes a,c) and A-485 (lanes b,d) were immunoprecipitated from 125I-labeled platelet lysate and analyzed after reduction (R), or not reduced (NR) as indicated.