Biochemical Characterization of VLA-1 and VLA-2

CELL SURFACE HETERODIMERS ON ACTIVATED T CELLS*

(Received for publication, May 17, 1985)

Martin E. Hemler, Jennie G. Jacobson, and Jack L. Strominger

From the Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

The very late antigen complexes VLA-1 and VLA-2 which appear on long-term activated human T cells have been characterized with respect to 1) subunit arrangement, 2) location of monoclonal antibody (MAB) binding sites, 3) carbohydrate content, and 4) protein homology. Cross-linking experiments showed that the VLA-1 complex is a heterodimer composed of an M, 210,000 subunit (α1) in acid-labile association with an M, 130,000 subunit (β). The VLA-2 complex is a heterodimer with an M, 165,000 subunit (α2) in base-labile association with the M, 130,000 β subunit. The subunits of VLA-1 (α1β) and VLA-2 (α2β) each appear to be arranged with 1:1 stoichiometry. The MAb A-1A5 has been shown to bind to an epitope on the common β subunit, consistent with its recognition of both the VLA-1 and VLA-2 heterodimers. On the other hand, MAB TS2/7 bound to an epitope of the α1 subunit, thus explaining the specific recognition of the VLA-1 heterodimer by TS2/7. Digestion of the α1, α2, and β subunits with neuraminidase and with endoglycosidase F revealed that each subunit contains substantial sialic acid and N-linked carbohydrate. By one-dimensional peptide mapping, the α1, α2, and β subunits were shown to be highly nonhomologous with respect to each other, although each subunit from different T cell sources appeared highly homologous if not identical.

Stimulation of resting T lymphocytes by antigen or mitogen results in T cell proliferation, accompanied by the appearance of immunocompetent cellular functions. At the same time, numerous cell surface glycoproteins are newly expressed and some, such as the IL-2 receptor (1, 2), 4F2 antigen (3, 4), transferrin receptor (5, 6), and insulin receptor (7), appear early, even before DNA synthesis (8). Other cell surface proteins such as HLA-DR (9–12), OKT10 (13, 14), and ACT-transferrin receptor (5, 6), and insulin receptor (7), appear of immunocompetent cellular functions.

Increased amounts within a few days after T cell activation results in T cell proliferation, accompanied by the appearance of immunocompetent cellular functions.
established from T acute lymphocyte leukemia patients (31). Immunoprecipitation of Indicated Cell Surface Proteins—Cells were labeled with 125I using Chloroglycoluril, and then 1% Nonidet P-40 (NP40) cell lysates were used for immunoprecipitation as previously described (24, 26). Immune complexes were adsorbed onto Staphylococcus aureus Cowan I strain, and 100 μl of the MAB 187.1 (S. aureus Cowan I; a gift from Dr. S. Alexander) was added for 12 h at 37 °C. Then 50-100 μl of the MAb 187.1 and washed, the S. aureus Cowan I pellet was suspended in 20 μl of 50 mM EDTA, 0.5% NP40, 1% β-mercaptoethanol, 0.1 M NaPO4 (pH 6.1). Endo F (0.1-1 μl of enzyme prepared as described (33) and generously provided by Dr. S. Alexander) was added for 12 h at 57 °C. Then 50-100 μl of SDS-sample buffer was added, and gel electrophoresis was carried out in the normal manner. Digestion with V8 protease from S. aureus (Miles) was carried out during gel electrophoresis in a second dimension as described by Cleveland et al. (34). After first dimension gel electrophoresis, gel slices containing α', α2, and β proteins were excised (using dansylated β-galactosidase as a marker) and incubated for 1-2 h in 2 changes of 1 mM EDTA, 0.2% SDS, 0.125 mM Tris-HCl, pH 6.8. Gel slices were then polymerized into the second dimension stacking gel (3-4 cm above running gel), and 0.5-20 μg of V8 protease in 50-100 μl of sample buffer was added to wells above the polymerized gel pieces. Sample buffer contained 1 mM EDTA, 0.2% SDS, 0.125 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.1% bromphenol blue. Gel electrophoresis was carried out uninterrupted for 12-15 h on 5-19 or 10-19% linear acrylamide gradients.

RESULTS

Heterodimeric Structure of VLA-1 and VLA-2—From previous results, it was shown that the M, 310,000, 255,000, and 130,000 proteins immunoprecipitated by MAB A-1A5 (26) could be separated into the independent structures called VLA-1 (M, 210,000/130,000) and VLA-2 (M, 165,000/130,000) (23, 24). These structures were hypothesized to be composed of subunits arranged in a heterodimeric fashion. To directly analyze the stoichiometry and subunit arrangements of VLA-1 and VLA-2, cross-linking experiments were carried out (Fig. 1). When both VLA-1 and VLA-2 were simultaneously immunoprecipitated by MAB A-1A5 after cross-linking with 6 μg/ml DSP two multimers were observed at M, approximately 310,000 and 255,000 (Fig. 1B). Notably, M, 310,000 is the approximate sum of the α2 and β proteins, whereas M, 255,000 is the approximate sum of the α2 and β proteins. When MAB TS2/7 was used to immunoprecipitate only the α2 and β proteins of VLA-1 after cross-linking, the M, 310,000 but not the 255,000 multimer was observed (Fig. 1D). When both α2 and β proteins of VLA-2 were cross-linked from the T lymphoblastoid cell line HSB (which does not express VLA-1) and immunoprecipitated with MAB A-1A5, the M, 255,000 but not the 310,000 multimer was observed (Fig. 2). The M, 255,000 and 310,000 proteins resulting from cross-linking of CTL proteins are also shown for comparison (Fig. 2). Thus, from the results in Figs. 1 and 2, it is established that the M, 310,000 protein multimer corresponds to the α2 and β proteins of VLA-1 and the M, 255,000 multimer corresponds to the α2 and β proteins of VLA-2. Furthermore, each heterodimer appears to exist predominantly with the α and β subunits in a 1:1 stoichiometry. In addition to the predominant cross-linked products at M, 310,000 and 255,000, a less distinct smear of higher M proteins was also observed, especially with 25 and 100 μg/ml DSP (Fig. 1B and Fig. 2). These presumably result from less specific protein associations occurring in the presence of excess cross-linker. No proteins were immunoprecipitated by the negative control MAB J-2A2 (Fig. 1B) and all of the cross-linked multimers were cleaved to yield only the monomeric subunits in the presence of reducing agent (Fig. 1A). The apparent M of the reduced subunits (Fig. 1A) is higher than that of the nonreduced subunits (Fig. 1B) perhaps due to the presence of interchain disulfide bonds, as previously noted (26). In contrast to the procedure used in Figs. 1 and 2 in which radiolabeled cell surface proteins were cross-linked after solubilization in NP40, cross-linking was also carried out on whole cells prior to solubilization. In these experiments, similar higher M multimers corresponding to cross-linked VLA-1 and VLA-2 were observed (data not shown). Subunit Localization of the MAB A-1A5- and TS2/7-Binding Epitopes—To further understand the structures of the VLA-1 and VLA-2 complexes, it was necessary to determine the subunit sites recognized by the MAB A-1A5 and TS2/7. Electrophoretic separation of the α2, α2', and β subunits followed by their transfer to nitrocellulose sheets by the technique of electroblotting (35) resulted in the loss of A-1A5- and TS2/7-binding epitopes. Thus, as an alternative, conditions of
varied pH were sought which would allow selective MAb binding to dissociated subunit components to identify those which contained specific MAb-binding sites. The immune complex containing VLA-1 immunoprecipitated with MAb TS2/7 was adsorbed onto Staphylococcus aureus Cowan I, and then washed using acidic and basic conditions prior to SDS-PAGE analysis (Fig. 3B) it was clear that MAb A-1A5, previously shown to recognize identical or overlapping epitopes (24), gave identical results, each subunit could be selectively retained while the untreated subunit was lost. At higher pH values, both subunits were retained in apparently equal ratios. When S. aureus Cowan I-immune complexes containing the MAb A-1A5 with VLA-1 and VLA-2 proteins were washed as described in the legend to Fig. 3A, inconclusive results were obtained (not shown). Alternatively, when radiolabeled extracts were treated at low pH for 30 min before neutralization and immunoprecipitation (Fig. 3B) it was clear that MAb A-1A5 could immunoprecipitate a2 and b proteins in the absence of the dissociated a2 subunit. Furthermore, the a2 subunit alone could be immunoprecipitated by MAb TS2/7. Thus the a2 subunit alone contained the TS2/7-binding epitope. MAB A-1A5 and MAB TS2/16, previously shown to recognize identical or overlapping epitopes (24), gave identical results, each precipitating a2 and b under acidic conditions. However, basic conditions readily caused these two subunits to dissociate (Fig. 4). When immune complexes containing A-1A5 and a2 and b proteins from two different T lymphoblastoid cell lines were washed at pH 10–10.8, the a2 subunit was lost and the b subunit was selectively retained. However, at pH 11.2 the b subunit was also lost. Thus, from Figs. 3 and 4 it is clear that the b subunit contains the A-1A5-binding site. Column chromatography using A-1A5 coupled to Sepharose also confirmed that the b subunit could be selectively retained while a2 was eluted at elevated pH (not shown). A weak M, 80,000 protein was present after washing at neutral pH but not at pH values at 10.0 or above (Fig. 4). Thus, this protein, previously seen in variable amounts (24, 26), appears not to bear the A-1A5-binding epitope.

Carbohydrate Content of the a2, a3, and b Subunits—The VLA-1 and VLA-2 protein complexes bind to ricin and lentil lectin (26), and thus at least one subunit of each is a glycoprotein. To determine the carbohydrate content of each subunit, digestions with neuraminidase and endoglycosidase F were carried out (Fig. 5). Neuraminidase (Fig. 5A, lanes b) caused a uniform decrease in the apparent M, of each subunit by M, 5,000–7,000 compared to untreated a2, a3, and b subunits from a long-term CTL line (lane a). Treatment of long-term PHA-stimulated T cells with Endo F (Fig. 5B, lanes b and c) resulted in a decrease of approximately M, 20,000 for the a3 subunit and M, 30,000 for the b subunit compared to untreated subunits (lane a). With longer autoradiographic exposure, it is seen that Endo F treatment (lanes e and f) also resulted in a decrease of about M, 10,000 for the weakly expressed a3 subunit compared to the untreated a3 subunit (lane d). Wide variability in the ratios of a3 to a2 proteins, such as seen in Fig. 5, A and B, has also been seen previously (23). The cell lines K-562 and U-937, which do not express

---

**Fig. 2.** Cross-linking of VLA-1 and VLA-2 proteins from a CTL line compared to VLA-2 from the T cell line HSB. 125I-radiolabeled cell extracts from HSB and a CTL line were cross-linked with DSP (see "Materials and Methods"), immunoprecipitated with MAb TS2/7, while the TS2/16 was selectively retained in the immune complex with the MAb A-1A5, and analyzed on SDS-PAGE analysis (Fig. 3). The cell lines K-562 and U-937, which do not express such as seen in Fig. 5, A and B, has also been seen previously (23). The cell lines K-562 and U-937, which do not express
the α1 or α2 subunits of VLA-1 and VLA-2, did express β subunits of variable size (Fig. 5C, lanes a and c). However, after removal of N-linked carbohydrate by Endo F, the unassociated β subunit from K-562 was reduced in size by M_

~33,000 (Fig. 5C, lane b) and from β of U-937 by M_

~42,000 (Fig. 5C, lane d). The resulting β subunits (M_

~100,000) are comparable in size to one another and to the Endo F-treated β subunits in Fig. 5B, lanes b, c, e, and f. A summary of the approximate size changes for the α1, α2, and β subunits from different sources after neuraminidase and Endo F treatment is shown in Table I.

VLA-1 and VLA-2: Distinct α1 and α2 Proteins with a Common β Subunit—To demonstrate that the α1, α2, and β proteins were distinct and highly nonhomologous, one-dimensional peptide mapping using V8 protease was carried out by the method of Cleveland et al. (34). Proteolytic digestion of the radiolabeled α1, α2, and β proteins from multiple activated T cell sources, using two different concentrations of V8 protease revealed (Fig. 6) that each protein was substantially nonhomologous to the others, because few, if any, peptides appeared to be shared between the α1, α2, and β subunits. The multiple peptide bands corresponding to V-8-digested β proteins were broad and indistinct compared to the 5–10 distinct fragments obtained from the α1 and α2 subunits. In this regard, the undigested M_

130,000 β protein from first-dimension electrophoresis has routinely been more broadly heterogeneous than the M_

210,000 and 165,000 α1 and α2 subunits. Possible explanations for the relatively indistinct β protein patterns could be that the β subunit is more hydrophobic and thus variably aggregated or more heterogeneous glycansylated relative to the α1 or α2 proteins. No apparent difference in the digestion patterns of the α1, α2, or β proteins from the T cells of one individual activated with alloantigen (CTL) and another activated with PHA was seen with either amount of protease digestion. Moreover, the α1 and α2 subunits of VLA-1 and VLA-2 from the transformed lymphoblastoid T cell lines C8215, ANITA, and HSB and the α1 and α2 subunits from long-term alloantigen-activated normal T cells yielded virtually identical V-8 protease digestion patterns (data not shown).

Because the β subunits found in both the VLA-1 and VLA-2 protein complexes are the same size (M_

130,000) and each is reactive with A-1A5, it was strongly suspected that a common β subunit is utilized. β subunits from T cell sources containing VLA-1 or VLA-2 or both VLA-1 and VLA-2 were
TABLE I
Estimated size changes resulting from neuraminidase and Endo F treatment of α', α2, and β subunits

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Long-term T cells</th>
<th>K-562</th>
<th>U-937</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated +Neuraminidase</td>
<td>+Endo F</td>
<td>Untreated +Endo F</td>
</tr>
<tr>
<td>α'</td>
<td>210,000</td>
<td>204,000</td>
<td>Absent</td>
</tr>
<tr>
<td>α2</td>
<td>165,000</td>
<td>160,000</td>
<td>Absent</td>
</tr>
<tr>
<td>β</td>
<td>130,000</td>
<td>123,000</td>
<td>100,000</td>
</tr>
</tbody>
</table>

FIG. 6. Comparison of α', α2, and β proteins by peptide mapping with V8 protease. Radiolabeled α', α2, and β proteins were immunoprecipitated using A-1A5, and then single vertical pieces of gel each containing all three proteins were excised, polymerized horizontally in another gel, and treated with either 2 μg (Part A) or 20 μg (Part B) of V8 protease during a second dimension electrophoresis (5–19% gradient) as described under "Materials and Methods." The SDS-PAGE separation results from the first dimension electrophoresis are shown at the top of each panel.

DISCUSSION
MAB A-1A5 was first used to define a complex series of cell surface proteins on T cells (M, 210,000, 165,000, and 130,000), with variable expression on different cells at different times (26), but the precise relationship between these proteins has not been understood. However, a second MAb, TS2/7, was used to distinguish among the A-1A5-reactive proteins and separated the VLA-1 complex (M, 210,000/130,000) from the VLA-2 complex (M, 165,000/130,000) (23, 24). In this paper, the MAb TS2/7 and A-1A5 have been used in a series of experiments involving cross-linking, epitope mapping, and biochemical characterization to establish more precisely the structure and arrangement of the VLA-1 and VLA-2 complexes. Some of these findings are summarized in schematic form in Fig. 8.

In cross-linking experiments, dithiobis(succinimidyl propionate) was used to demonstrate that VLA-1 is composed of the subunits α' and β whereas VLA-2 is composed of the associated α2 and β subunits. Also, it appeared from the size of the cross-linked multimers that the subunits of both VLA-1 and VLA-2 are perhaps primarily arranged with 1:1 stoichi-
the VLA-1 and VLA-2 were immunoprecipitated using MAb A-1A5 and analyzed in the first dimension by SDS-PAGE (8% acrylamide gradient) and treated with 1 or 10 μg of V8 protease in the gel. This second dimension analysis of the peptide fragments was carried out on a 10–19% acrylamide gradient. Sources of 125I-lale VLA-1 and/or VLA-2 were the T lymphoblastoid cell line ANITA (expressing mostly VLA-2, lane A), the T lymphoblastoid cell line C8215 (expressing VLA-1, lane B), and long-term in vitro cultured CTL (expressing VLA-1 and VLA-2, lanes C and D). The V8 in lane D is the same as that in lane C except that VLA-1 has been previously removed by a preimmunoprecipitation step using MAb TS2/7 which recognizes only VLA-1. The cell lines ANITA and C8215 (provided by Dr. W. Haseltine) are transformed due to the presence of part of the HTLV-1 genome.

Fig. 7. Comparison of V8 protease peptides from the β subunits of VLA-1 and VLA-2. VLA-1 and/or VLA-2 were immunoprecipitated using MAb A-1A5 and analyzed in the first dimension by SDS-PAGE (8% acrylamide). Gel pieces corresponding to only the β subunit were excised, polymerized into the stacking gel portion of a second acrylamide gel (see “Materials and Methods”) and treated with 1 or 10 μg of V8 protease in the gel. This second dimension analysis of the peptide fragments was carried out on a 10–19% acrylamide gradient. Sources of 125I-labeled VLA-1 and/or VLA-2 were the T lymphoblastoid cell line ANITA (expressing mostly VLA-2, lane A), the T lymphoblastoid cell line C8215 (expressing VLA-1, lane B), and long-term in vitro cultured CTL (expressing VLA-1 and VLA-2, lanes C and D). The V8 in lane D is the same as that in lane C except that VLA-1 has been previously removed by a preimmunoprecipitation step using MAb TS2/7 which recognizes only VLA-1. The cell lines ANITA and C8215 (provided by Dr. W. Haseltine) are transformed due to the presence of part of the HTLV-1 genome.

Fig. 8. Subunit structure of VLA-1 and VLA-2 and localization of MAb-binding epitopes.

ometry and thus are heterodimers. The discovery that VLA-1 and VLA-2 are heterodimers, together with the mapping of the TS2/7-binding site to the α1 protein and the mapping of the A-1A5-binding site to the β subunit, now allows us to understand the immunoprecipitation of only VLA-1 by TS2/7 and the immunoprecipitation of both VLA-1 and VLA-2 by A-1A5. Fig. 5 also emphasizes that A-1A5 can recognize the free β subunit, unassociated with α or α2 proteins such as found on unactivated T cells (24, 26). In this regard, it is important to note that immunoprecipitates from unactivated T cells occasionally have the sensitivity to show weak but distinct α and α2 protein bands, even when the α1 protein is not detectable by less sensitive cell surface analysis using the MAb TS2/7. However, on unactivated T cells the β subunit is largely unassociated with α or α2.

For the method of assignment of the A-1A5- and TS2/7-binding epitopes to the β and α1 subunits, respectively, it was required: 1) that the “other” subunit not be needed to maintain the integrity of the MAb binding epitope; and 2) that conditions be found whereby the “other” subunit could be eluted without disrupting the binding between the MAb and the binding epitope. A variety of conditions involving changes in pH or addition of heat, detergent, or chemical reduction have been used to map successfully the MAb-binding epitopes on other multisubunit proteins such as LFA-1 and MAC-1 (36, 37), the 4F2 antigen (4), and the T cell receptor (38). In this study, changes in pH not only selectively removed the “uninvolved” subunits but also revealed that subunit associations in VLA-1 are acid labile whereas VLA-2 is base labile.

The results from the one-dimensional peptide-mapping experiments now confirm that the α1, α2, and β subunits are nonhomologous at the protein sequence level. Other evidence consistent with this nonhomology is that α1, α2, and β proteins have: 1) different sizes, even after the removal of N-linked glycans (Fig. 5, Table I); 2) distinct non-cross-reactive MAb-binding epitopes (summarized in Fig. 8); 3) different cell distributions for each subunit (24, 26); and 4) apparently different chromosomal map locations for the α1 and β subunits (27). On the other hand, these different subunits on T cells from different individuals, activated by different stimuli or from malignant or normal cells, appear highly homologous, if not identical, and the same β subunit appears to be present in both VLA-1 and VLA-2. However, while one-dimensional V8 protease mapping can clearly establish nonhomology and/or suggest homology, it must be realized that only a small number of peptides have been analyzed by this technique. Thus, homology of some sequences between α subunits and nonhomology between β subunits may be overlooked by this method. Absolute proof of homology or nonhomology awaits more detailed analysis at the protein or DNA sequence level.

The structures of VLA-1 and VLA-2, with different α subunits sharing a common β subunit, thus have some analogy to the arrangement of the LFA-1, MAC-1 family of proteins in mouse (36) and human (38). In that protein family in humans, there appear to be three distinct α subunits of M, 180,000, 170,000, and 160,000 which are each in heterodimeric arrangement with a common β subunit of M, 95,000 (38). Furthermore, separate immunologic functions for the mouse and human LFA-1 and MAC-1 heterodimers have been identified (36, 38). By analogy, then, the possibility exists that the VLA-1/VLA-2 family of proteins may also eventually be
discovered to be associated with multiple physiological functions. Another complex of multiple proteins in the M<sub>120,000–170,000 region has been defined by Mabs A22 and Ab143 (39, 40). However, it has not yet been established if these proteins are arranged as subunit multimers. Furthermore, unlike the A-1A5-reactive β subunit, these widely distributed human cell surface antigens may be missing from spleen and lymph node (40). Other recently characterized cell surface high M<sub>105,000 Tα (20) and gp115 (41). However, no associated subunits have been described for these structures, and unlike VLA-1 β subunits, gp115 increases in apparent M<sub>120,000 when desialylated.

The current results using Endo F help to explain the basis for the increased size previously seen for the A-1A5 subunit and another cell surface protein, the heavy chain of 4F2 antigen (4) have the same patterns of N-linked glycosylation. In both cases, they are more highly glycosylated on the monocytoid lines U-937 and HL-60 compared to the erythroid line K-562 and T cell lines such as HSB. Thus, there may be a uniform cell type-specific difference in N-glycosylation machinery which leads to the increased N-glycosylation of multiple unrelated cell surface proteins on cell lines such as U-937 and HL-60.

The structural information provided in this paper should provide a useful basis of understanding from which later studies such as purification and gene cloning can be carried out. Furthermore, understanding the structural arrangement of VLA-1 and VLA-2 will facilitate future studies investigating possible immunoregulatory functions of these molecules. In this regard, it has recently been shown that the ratio of VLA-1 expression can sometimes be regulated in a manner inverse to IL-2 receptor expression (42).

REFERENCES