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Structure–function analysis of the human integrin VLA-4 (α4/β1)

Correlation of proteolytic α4 peptides with α4 epitopes and sites of ligand interaction

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The structure–function relationship of the human integrin VLA-4 (α4/β1; CD49d/CD29), has been studied in the human B-cell line Ramos by immunochemical and functional analysis. Ramos cells expressed the 150-kDa non-proteolyzed form of the α4 chain, which could be digested upon mild trypsin treatment to generate the 80- and 65-kDa proteolyzed forms, as well as α4 polypeptides of 55 and 50 kDa. In addition, treatment of Ramos cells with high doses of pronase predominantly yielded the 55- and 50-kDa α4 peptides. The trypsin-generated 80- and 65-kDa α4 polypeptides, but not the 55- and 50-kDa fragments, were able to associate with the β1 chain. Distinct anti-VLA-4 mAb against four different α4 epitopes, referred to as epitopes A, B1, B2, and C, recognized the 150-kDa α4 chain both associated or non-associated with the β1 chain. The α4 proteolytic forms of 80, 65 and 50 kDa were precipitated by the anti-α4 mAb directed against the four different α4 epitopes. On the other hand, the 55-kDa α4 peptide was present in precipitates from anti-α4 mAb specific for epitopes A, B1 and C, but absent in precipitates from the anti-α4 mAb specific for epitope B2. The different adhesive capacities of the VLA-4 integrin, namely the interaction with a 38-kDa fibronectin fragment containing the CS-1 region of plasma fibronectin (Fn-38), the binding to the vascular cell adhesion molecule-1 (VCAM-1), or the ability to mediate the anti-α4-induced cell aggregation, were not altered on VLA-4 from cells upon mild trypsin treatment, when compared to non-treated cells. However, the 55- and 50-kDa α4 forms generated by high-dose pronase cell treatment, failed to mediate cell interaction with Fn-38 or VCAM-1 ligands, and cell aggregation could not be triggered through VLA-4 under these conditions.

VLA antigen; Integrin; Leukocyte adhesion

1. INTRODUCTION

The VLA-4 integrin (α4/β1; CD49d/CD29) is a leukocyte glycoprotein involved in both cell–extracellular matrix and cell–cell interactions [1,2]. VLA-4 is the leukocyte receptor for the CS-1 region of plasma fibronectin [3–5], as well as for the vascular cell adhesion molecule VCAM-1 [6,7], and also mediates cell aggregation through an LFA-1/ICAM-1 independent mechanism [8,9]. The α4/β1 VLA-4 heterodimer is composed of a chain of 150 kDa (α4 chain) non-covalently associated to a subunit of 130 kDa (β1) [10,11]. The 150-kDa α4 subunit can be proteolyzed 'in vivo' or 'in vitro' to generate α4 polypeptides of 80 and 65 kDa [10–12], although the physiologic significance of this proteolysis is unknown. We have defined four distinct epitopes on the VLA-α4 chain (A, B1, B2, and C) by both immunological and functional criteria [13]. Anti-epitope A, B1, and B2 mAb directly affected the adhesive cell interactions mediated by VLA-4, whereas the anti-epitope C mAb did not [13]. Here we have studied the correlation of the presence of α4 proteolytic peptides with the distinct α4 epitopes and with the VLA-4 sites of ligand interaction.

Abbreviations: VLA, very late activation antigen; mAb, monoclonal antibody(ies); Fn, fibronectin; VCAM-1, vascular cell adhesion molecule-1; LFA-1, lymphocyte-function associated antigen-1; ICAM-1, intercellular adhesion molecule-1.

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2. MATERIALS AND METHODS

2.1. *Cells and protease treatment*

The human B-cell line Ramos, derived from a Burkitt lymphoma, was obtained from the American Type Culture Collection (Rockville, MD), and was grown in RPMI 1640 medium (Flow Laboratoires, Irvine, Scotland) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamicine. For trypsin treatment, radiolabeled cells were incubated (5×10^6/ml) in trypsin EDTA 1× (Flow Laboratoires) for 3 min at 37°C. For Pronase treatment, radiolabeled cells were incubated in PBS (5×10^6/ml) with 20 μg/ml or 100 μg/ml pronase (Sigma, St. Louis, MO) for 30 min at 37°C. After proteolysis, cells were washed and lysed, and lysates were subjected to immunoprecipitation.

2.2. *Monoclonal antibodies, fibronectin fragments and soluble VCAM-1*

The anti-α4 HP1/7 (epitope A), HP2/1 (epitope B1), HP2/4 (epitope B2), and B-5G10 (epitope C) mAb, and the anti-β1 TS2/16 mAb, have been described previously [10,11,13,14]. The 38-kDa Fn fragment was prepared by tryptic digestion of human plasma Fn as described [15]. The recombinant soluble form of VCAM-1 was purified by immunoaffinity chromatography from conditioned medium of CHO cells stably transfected with a truncated cDNA for VCAM-1 (Lobb, R., et al., manuscript submitted).

2.3. *Radiolabeling, immunoprecipitation and electrophoresis*

Cell suspensions were cell-surface radioiodinated in solution with chloroglycoluril (Iodogen, Pierce Chemical Co., Rockford, IL). After protease treatments, cells were lysed in PBS pH 7.4, 1% Triton X-100, 1% hemoglobin, and 1 mM PMSF ($\alpha 4/\beta 1$ association conditions), or in PBS pH 10.5, 2% Triton X-100, 1% hemoglobin, 0.3 M NaCl, 2 mM EDTA, and 1 mM PMSF ($\alpha 4/\beta 1$ dissociation conditions). For immunoprecipitation, the ^{125}I-labeled proteins were mixed with 100 μl of mAb-containing culture supernatants, followed by 100 μl of 187.1 anti-mouse kappa chain and 30 μl of protein A from *S. aureus* coupled to Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Immunoprecipitates were processed as described [10], and samples were subjected to SDS-(10% acrylamide) PAGE and autoradiography.

2.4. *Cell attachment to fibronectin and VCAM-1 and aggregation assays*

For cell-attachment assays, 96-well plates were coated with 100 μl of 0.1 M $NaHCO_3$ containing 10 μg/ml of 38-kDa Fn fragment or 5 μg/ml of recombinant soluble VCAM-1, as described [13]. Then, cells (2×10^6/ml) were resuspended in RPMI 1% BSA and plated in duplicate on the coated plates (100 μl final volume). After 30 min of incubation at 37°C, unbound cells were removed by washing, and bound cells were quantified by counting the cells from at least three different fields of known area. The number of cells on a non-washed well was referred as input cells (100% of binding). For antibody inhibition of cell attachment, cells were preincubated with 1:10 final dilution of mAb-containing culture supernatants before the adhesion assay. Homotypic aggregation assays were performed as described [8]. Cells were incubated (2×10^6/ml) in duplicate in RPMI 5% fetal calf serum in the presence of 1:10 final dilution of mAb-containing culture supernatants (100 μl final volume), for 3 h at 37°C and 5% CO_2 into a cell incubator, and aggregation was determined by visualization of the plate with an inverted microscope. Percent aggregation was measured by the following equation: percent aggregation = 100 × (1 − (number of free cells)/(total number of cells)).

3. RESULTS

Four distinct epitopes (A, B1, B2 and C) have been defined previously on the VLA-$\alpha 4$ subunit, which show different immunological and functional properties [13]. To analyze the topographic location on the $\alpha 4$ chain of these different antigenic sites, immunoprecipitation experiments on the B-lymphoblastoid cell line Ramos were carried out by using mAb recognizing the four distinct $\alpha 4$ epitopes. Ramos cells were cell-surface radioiodinated and lysed either under conditions which kept the $\alpha 4/\beta 1$ heterodimer associated (Fig. 1A) or dissociated (Fig. 1C, lanes 1–5), and then, the lysates were subjected to immunoprecipitation with the distinct anti-VLA-4 mAb. As observed, the mAb directed against the four different $\alpha 4$ epitopes recognized the 150-kDa $\alpha 4$ subunit both associated to (A, lanes 1–4) or dissociated from (C, lanes 1–4) the 130-kDa $\beta 1$ subunit, which was also recognized under both conditions by an anti-$\beta 1$ mAb (A and C, lanes 5).

Next, radioiodinated cells were incubated under mild trypsin treatment conditions, lysed upon $\alpha 4/\beta 1$ association (Fig. 1B, lanes 3–4) or dissociation (Fig. 1C, lanes 6–10) conditions, and precipitated with the anti-$\alpha 4$ or anti-$\beta 1$ mAb. $\alpha 4$ polypeptides of 80, 65, 55 and 50 kDa were produced by the trypsin treatment and precipitated by an anti-$\alpha 4$ mAb (HP2/1, epitope B1), in association with the $\beta 1$ component (B, lane 3). When samples were precipitated with an anti-$\beta 1$ mAb, only the 80- and 65-kDa $\alpha 4$ forms, but not the 55- and 50-kDa forms, were able to associate with the $\beta 1$ subunit, which showed a proteolyzed form of 120 kDa under these conditions (B, lane 4). In Fig. 1B, lanes 1 and 2, precipitates from control cells, using the anti-$\alpha 4$ HP2/1 and anti-$\beta 1$ TS2/16 mAb, respectively, are shown. The four distinct anti-$\alpha 4$ mAb precipitated the 80-, 65-, and

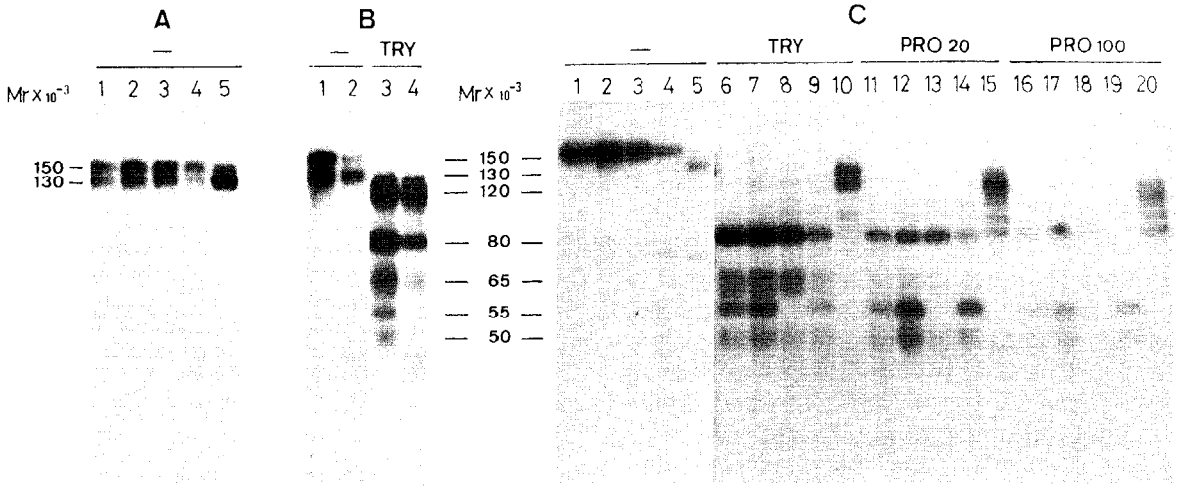

Fig. 1. Immunoprecipitation of $\alpha 4$ proteolytic polypeptides with distinct anti-$\alpha 4$ mAb upon $\alpha 4/\beta 1$ association or dissociation conditions. (A) Radioiodinated Ramos cells were lysed under $\alpha 4/\beta 1$ association conditions, and lysates were precipitated with anti-$\alpha 4$ HP1/7 (lane 1), HP2/1 (lane 2), HP2/4 (lane 3) and B-5G10 (lane 4) mAb, or with anti-$\beta 1$ TS2/16 (lane 5) mAb. (B) Radioiodinated cells were maintained non-treated (lanes 1,2) or were treated with trypsin (lanes 3,4). Cells were lysed under $\alpha 4/\beta 1$ association conditions, and precipitated with anti-$\alpha 4$ HP2/1 (lanes 1 and 3) and anti-$\beta 1$ TS2/16 (lanes 2 and 4) mAb. (C) Radioiodinated cells were maintained non-treated (lanes 1–5), or were incubated with trypsin (lanes 6–10), 20 μg/ml pronase (lanes 11–15), or 100 μg/ml pronase (lanes 16–20) as described in section 2. Cells were lysed upon $\alpha 4/\beta 1$ dissociation conditions, and lysates were preincubated with anti-$\alpha 4$ HP1/7 (lanes 1,6,11 and 16), HP2/1 (lanes 2,7,12 and 17), HP2/4 (lanes 3,8,13 and 18), and B-5G10 (lanes 4,9,14 and 19) mAb, or with anti-$\beta 1$ TS2/16 (lanes 5,10,15 and 20) mAb. Immune complexes were isolated, and reduced samples were subjected to SDS-(10% acrylamide) PAGE and autoradiography.

Table I

Functional properties of α4 proteolytic polypeptides

Cell treatment[a]	α4 polypeptides (kDa)	Cell binding to[b]				Induction of cell[b] aggregation by	
		Fn-38	Fn-38 + HP2/1	VCAM-1	VCAM-1 + HP2/1	HP1/7	HP2/4
None	150	90±6	2±1	92±6	2±1	41±17	52±16
Trypsin	80,65,55,50	82±16	3±2	83±10	4±2	51±15	66±13
Pronase (20 μg/ml)	(80,65),55,50	10± 4		6±2		4±1	6±3
Pronase (100 μg/ml)	55,50	2±1		2±1		2±1	2±1

[a]Ramos cells were treated with proteases as described in section 2.

[b]Cell attachment to Fn-38 of VCAM-1 coated-plates was analyzed by 'in vitro' cell adhesion assays, in the presence or absence of anti-α4 HP2/1 (epitope B1) mAb, as described in section 2. Results are expressed as mean ±SE of percentage of binding, calculated from three separated experiments.

[c]Induction of homotypic cell aggregation with anti-α4 (epitope A) or HP2/4 (epitope B2) mAb was analyzed by 'in vitro' aggregation assays, as described in section 2. Results are expressed as mean ±SE of percentage of cell aggregation, calculated from three separated experiments.

50-kDa α4 peptides (C, lanes 6–9). However, the 55-kDa form was precipitated by the anti-epitope A, anti-epitope B1, and anti-epitope C mAb (C, lanes 6, 7 and 9), but not by the anti-epitope B2 mAb (C, lane 8).

Proteolytic treatment of radioiodinated Ramos cells was also performed by cell incubation with pronase (Fig. 1C, lanes 11–20). Under low-dose pronase treatment, the relative amount of both the 80- and 65-kDa forms, with respect to the 55- and 50-kDa forms, decreased considerably, when compared to the trypsin-treated cells (C, lanes 11–14 vs. lanes 6–9). When a high dose of pronase was employed, the 55- and 50-kDa peptides were predominantly generated, and both of them precipitated by the anti-epitope A, anti-epitope B1, and anti-epitope C mAb (C, lanes 16, 17 and 19), whereas the anti-epitope B2 mAb only precipitated the 50-kDa form (C, lane 18), similarly to that observed upon the trypsin incubation conditions.

To study the structure–function relationship on the VLA-4 integrin, the cell adhesion processes mediated by VLA-4 were investigated on both trypsin- and pronase-treated Ramos cells by 'in vitro' functional assays (Table I). The VLA-4 interaction either with a 38-kDa plasma fibronectin fragment containing the CS-1 region (Fn-38), or with a recombinant soluble form of VCAM-1, as well as the capability to aggregate upon incubation with aggregation inducer anti-α4 mAb, were unaffected on cells treated with trypsin under mild conditions, as compared with untreated cells. As shown, the cell interaction with Fn-38 or VCAM-1 was completely blocked in the presence of the anti-α4 HP2/1 (epitope B1) mAb, both in control or trypsin-treated cells. By contrast, cell treatment with pronase resulted in the abrogation of the three VLA-4-mediated adhesion activities (Table I), indicating that, upon the conditions in which the 55- and 50-kDa α4 forms are mainly present, VLA-4 has no functional activity.

4. DISCUSSION

In this study, we have found a pattern of proteolytic degradation of the α4 protein composed of polypeptides of 80-, 65-, 55- and 50-kDa, which showed different functional properties and reacted differentially with anti-α4 mAb defining the distinct α4 epitopes (Table II). The results reported here demonstrate the existence of biochemical differences in the α4 epitopes previously defined on the basis of immunological and functional criteria [13]. In this respect, it is remarkable that we found that anti-epitope B1 and anti-epitope B2 mAb, known to cross-compete with each other [13], show different functional properties in terms of induction of cell aggregation and that they precipitate distinct α4 proteolytic fragments. By contrast, the anti-epitope A, anti-epitope B1, and anti-epitope C mAb, which have distinct immunological and functional properties [13], precipitated the same α4 proteolytic pattern (Table II).

The proteolytic 'in vivo' degradation of the VLA-α4 subunit has been described, and appears to be regulated in a cell-type specific manner [2,10,11]. However, the functional meaning of this cleavage remains undiscovered. Our results indicate that the 80- and 65-kDa α4 forms, proteolyzed 'in vitro' on Ramos cells, have functional and immunological properties identical to the native 150-kDa protein. The exact location of the distinct α4 epitopes on the different α4 proteolytic polypeptides is hampered by the fact that the 80- and 65-kDa polypeptides remain non-covalently associated after proteolysis [11,12]. Interestingly, the 80- and 65-kDa α4 polypeptides, but not the 55- and 50-kDa forms, were able to associate 'in vitro' with the β1 chain and mediate all the cell adhesion functions due to the VLA-4 integrin. From our results, we hypothesize that the 80- and 65-kDa α4 peptides can be further proteolyzed to generate the 55- and 50-kDa peptides, which cannot be

Table II

Immunological, biochemical and functional properties of α4 intact and proteolytic peptides

α4 polypeptides (kDa)	Precipitation by anti-α4 mAb				Association with β1 chain	Function[b]		
	A	B1	B2	C[a]		Fn-38	VCAM-1	Aggregation
150	+	+	+	+	+	+	+	+
80,65	+	+	+	+	+	+	+	+
55	+	+	–	+	–	–		–
50	+	+	+	+	–	–		–

[a] α4 epitopes A, B1, B2 and C are as described in ref. [13].
[b] VLA-4 interaction with Fn-38 or VCAM-1, or induction of cell aggregation by the anti-α4 HP1/7 (epitope A) or HP2/4 (epitope B2) mAb.

associated to the β1 chain. Thus, this additional proteolysis of the 80- and 65-kDa α4 fragments would produce the loss of VLA-4 cell-adhesion functions, and also the abrogation of epitope B2 on the 55-kDa fragment.

Similarly to other integrins, the cell adhesion functions mediated by VLA-4 are dependent of the presence of divalent cations [1]. Some anti-β2 or anti-β3 integrins mAb, related to the integrin function, recognize divalent cation-dependent epitopes [16–18]. However, all α4 epitopes recognized by the distinct anti-α4 mAb here studied, including those which trigger cell aggregation and/or inhibited VLA-4 interaction with Fn or VCAM-1, were not dependent on the presence of divalent cations (Fig. 1C; and data not shown). Furthermore, the anti-α4 mAb recognized the α4 chain independently of its association with the β1 chain. The VLA-4 integrin is exclusively expressed on leukocytes, and constitutes the molecule through which these cells interact with the splicing-regulated IIICS region of Fn, and also with activated endothelium via VCAM-1 [3–5,7]. In addition, the existence of other unknown VLA-4 cellular ligands has been proposed [2,13]. The Fn amino-acid sequences which interact with VLA-4 have been identified [4,19]. Thus, the finding and characterization of the distinct ligand binding sites on VLA-4 at a molecular level will be of crucial importance in order to design reagents which can interfere selectively with different VLA-4 mediated adhesion functions. These can be potential therapeutic tools for the use in pathological processes involving leukocyte recognition of extracellular matrix proteins and migration towards inflamed tissues.

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