Expression of glycophosphatidylinositol-anchored and -non-anchored isoforms of vascular cell adhesion molecule 1 in murine stromal and endothelial cells

Tatsuo Kinashi, Yves St. Pierre, and Timothy A. Springer
The Center for Blood Research, Harvard Medical School, Boston, Massachusetts

Abstract: Monoclonal antibodies to murine vascular cell adhesion molecule-1 (VCAM-1, CD106) revealed not only the expected VCAM-1 molecule with an apparent molecular weight of 100 kDa, but also a molecule with a smaller size of 46 kDa in stromal cells and stimulated endothelial cells. Peptide mapping suggested the 46 kDa and 100 kDa proteins were closely related. The 46 kDa, but not 100 kDa protein, was cleaved from the cell surface with phosphatidylinositol-specific phospholipase C (PI-PLC), showing that the 46 kDa protein was a GPI-linked molecule. The 46 kDa and 100 kDa isoforms of VCAM-1 were shown to be *N*-glycosylated, have similar kinetics of biosynthesis, and to be partially shed from the cell surface with a slight reduction of size. TNF-α induced both isoforms of VCAM-1 with a similar time course of appearance on the surface of endothelial cells. The relative amounts of the 46 kDa and 100 kDa isoforms depended on the cell type examined. The GPI-anchored isoform is functionally important, because on a cell on which it was expressed almost as well as the 100 kDa isoform, treatment with PI-PLC reduced VLA-4-dependent conjugate formation. *J. Leukoc. Biol.* 57: 168–173; 1995.

Key Words: *cell-cell adhesion • tumor necrosis factor α • very late antigen-4 • CD49d/CD29 • CD106*

INTRODUCTION

Vascular cell adhesion molecule-1 (VCAM-1) is an integral membrane protein that belongs to the immunoglobulin (Ig) superfamily. VCAM-1 is expressed in cytokine-stimulated endothelial cells [1], bone marrow stromal cells [2, 3], and myoblasts [4]. The counter-receptor for VCAM-1 is the integrin very late antigen-4 (VLA-4, CD49d/CD29) [1, 5]. The interaction between VCAM-1 and VLA-4 has been shown to be important in lymphocyte-endothelial cell adhesion, lymphopoiesis [2, 3], cytotoxic T cell killing [6], germinal center development [7], tumor metastasis [8, 9], atherogenesis [10], acute graft rejection [11], and the formation of myotubes [4].

Human VCAM-1 can be alternatively spliced, giving rise to molecules containing six or seven Ig-like domains; however, the six-domain form is only expressed in trace amounts [1, 12–14]. Both isoforms have a classical type I transmembrane polypeptide chain anchor. Since VLA-4 can bind both domain 1 and the alternatively spliced domain 4 of VCAM-1, alternative forms of VCAM-1 may represent a mechanism for regulating the number of integrin binding sites per molecule or the density of integrin binding sites on the surface of endothelium [15, 16]. Murine VCAM-1 has been characterized as a 100 kDa molecule by immunoprecipitation and cDNA cloning has revealed seven Ig-like domains and a transmembrane polypeptide chain anchor [17].

During investigations on the role of VCAM-1 in leukocyte-endothelial cell and hematopoietic cell-stromal cell interactions in mice, we identified a glycophosphatidylinositol (GPI)-anchored isoform of VCAM-1 of 46 kDa which we term gp46. gp46 is expressed in amounts less than or comparable to gp100 in different cell types and on some cell types is functionally important in adhesion to VLA-4, as shown by release by phosphatidylinositol-specific phospholipase C (PI-PLC).

MATERIALS AND METHODS

Cell lines

A stromal cell line, PA6 [18], was maintained in RPMI 1640 containing 10% fetal calf serum (FCS), 50 μM β-mercaptoethanol, and 50 μg/ml gentamycin (complete medium). The endothelial cell lines, E-End-2 and S-End-1 [19, 20], were maintained in complete medium without β-mercaptoethanol. A hematopoietic precursor cell line, LyD9 [21], was cultured in complete medium supplemented with 10% WEHI-3B conditioned medium as a source of IL-3.

Antibodies, growth factors, and other materials

Two anti-murine VCAM-1 monoclonal antibodies (mAbs), MVCAM-A (clone 429) and MVCAM-B (clone 584), (rat IgG2a) were produced as described [22] by using PA6 as immunogen, and screening for inhibition of VLA-4-dependent adhesion of LyD9 to PA6. These mAbs block adhesion mediated by VLA-4 to purified VCAM-1, as will be described in detail elsewhere. The anti-murine VLA-4 mAb, PS/2 (rat IgG2a), the anti-murine VCAM-1 mAb, M/K-1.9 (rat IgG1) [2, 3], and the anti-murine LFA-1 mAb, M17/4.4.11.9 [23] have been described. M/K-1.9 and M/K-2 mAb have the same specificity for VCAM-1 and cross-block one another [3]. All mAbs were purified by protein G fast flow Sepharose chromatography (Pharmacia, Piscataway, NJ) except M/K-1.9 which was used as a culture supernatant. Purified mouse tumor necrosis factor α (TNF-α) was purchased from

Abbreviations: BSA, bovine serum albumin; FCS, fetal calf serum; GPI, glycophosphatidylinositol; Ig, immunoglobulin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen-4.

Reprint requests: Timothy A. Springer, The Center For Blood Research, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115.

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Genzyme (Boston, MA). PI-PLC (600 U/mg) from *Bacillus cereus* was purchased from Boehringer Mannheim (Indianapolis, IN).

Conjugation assay [24]

PA6 and TNF-α-stimulated E-End-2 cells were suspended with 5 mM EDTA in phosphate-buffered saline (PBS), pH 7.4, for 5 min at 20°C, washed, resuspended in RPMI 1640/10% FCS, and labeled by incubating the cells with 50 μg/ml sulfofluorescin diacetate (Molecular Probes, Eugene, OR) for 45 min at 37°C. LyD9 was labeled with 40 μg/ml hydroethidine (Polysciences, Warrington, PA) for 45 min at 37°C. LyD9 cells (1.5×10^6/150 μl) were mixed with labeled PA6 or E-End-2 cells (5×10^5/250 μl) and incubated for 30 min at 37°C with or without antibodies. Conjugate formation was measured using a FACScan (Becton Dickinson, Rutherford, NJ). Results were expressed as the percentage of PA6- or E-End-2-forming conjugates with LyD9 cells. For PI-PLC treatment, the labeled PA6 or E-End-2 cells were incubated in PBS containing 0.5% glucose, 0.5% bovine serum albumin (BSA), and 0.1 mM EDTA with or without 100 mU/ml PI-PLC at 37°C for 1 h. Cells were washed and resuspended in RPMI 1640 containing 10% FCS.

Cell surface labeling and immunoprecipitation

Cells were surface labeled with IodoGen (Pierce Chemical Co., Rockford, IL) and $Na^{125}I$ as described [25]. Cell lysates were obtained by resuspending the cells with the lysis buffer containing 150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, soybean trypsin inhibitor (20 μg/ml), leupeptin (20 μg/ml), pepstatin A (1 mM), aprotinin (0.1 TIU/ml), and PMSF (1 mM) for 1 h at 4°C. After centrifugation, the cell lysates were precleared with Pansorbin (Calbiochem, La Jolla, CA), and subjected to immunoprecipitation with MVCAM-A conjugated to Sepharose 4B (10 mg/ml; Pharmacia) or rat IgG-Sepharose 4B as control. Immunoprecipitated proteins were subjected to SDS 10% PAGE, followed by autoradiography at −80°C with intensifying screens.

Peptide mapping with V8 protease

After separation of immunoprecipitates with MVCAM-A from ^{125}I-labeled cell lysates by SDS-PAGE, gel slices corresponding to the 100 kDa and 46 kDa bands were excised with an autoradiogram as a template. Gel slices were digested with V8 protease from *S. aureus* (Sigma Chemical Co., St. Louis, MO) as described [26] and subjected to SDS 15% PAGE.

Analysis of glycosylation of VCAM-1

To examine glycosylation of VCAM-1, neuraminidase (Calbiochem), *N*-glycanase (Genzyme, Cambridge, MA), and *O*-glycanase (Genzyme) were used to digest VCAM-1 immunoprecipitated from ^{125}I-labeled PA6 following the manufacturer's instructions. The enzymatic reactions were stopped by the addition of the SDS-sample buffer and subjected to SDS-PAGE.

Treatment with PI-PLC

^{125}I-surface labeled cells (7×10^6) were incubated in 1 ml PBS containing 0.5% glucose, 0.5% BSA, and 0.1 mM EDTA with or without 100 mU/ml of PI-PLC, and incubated at 37°C for 1 h. Cells were then centrifuged at 4000 rpm in a microfuge and the pellets were resuspended in the lysis buffer. The supernatants were recentrifuged at 14,000 rpm for 10 min to remove cellular components. Both supernatants and cell lysates were immunoprecipitated with MVCAM-A and analyzed by SDS-PAGE.

Kinetics of VCAM-1 biosynthesis

PA6 cells were pulse-labeled in RPMI 1640 medium containing 5% dialyzed FCS and 0.5 mCi ^{35}S-methionine and cysteine (EXPRE^{35}S^{35}S, Protein Labeling Mix, NEN, Boston, MA) for 20 min and chased for varying times with complete medium containing excess methionine and cysteine. Preparation of cell lysates and immunoprecipitation with MVCAM-A were as described above. For the study of induction of VCAM-1 on E-End-2 cells, monolayers of cells in 10 cm dishes were incubated with 400 units/ml of TNF-α for varying times followed by surface-labeling with 0.5 mCi ^{125}I using lactoperoxidase and glucose oxidase (Sigma) for 10 min [27]. Preparation of cell lysates and immunoprecipitation with MVCAM-A were as described above.

RESULTS

By screening for inhibition of binding of the LyD9 hematopoietic progenitor cell line to the PA6 stromal cell line, two rat mAbs, designated MVCAM-A and MVCAM-B, were produced to mouse VCAM-1. These mAbs immunoprecipitated a major band with a mass of about 100 kDa (gp100) and a minor band of about 46 kDa (gp46) from the PA6 stromal cell line (**Fig. 1A,** lanes 3 and 4). The same pattern of immunoprecipitation was obtained with the M.K1.9 mAb of Miyake et al. [2] (Fig. 1A, lane 2), which had previously been used to identify gp100 as murine VCAM-1.

The amount of gp46 was found to vary depending on the cell type. Two mouse endothelial cell lines were stimulated with TNF-α and subjected to immunoprecipitation. The E-End-2 cell line expressed a high proportion of gp46 relative to gp100 (Fig. 1A, lane 6). However, another endothelial cell line, S-End-1 expressed gp100 with little, if any, gp46 (Fig. 1A, lane 7).

To determine whether gp46 was a distinct molecule or was related to gp100, we performed peptide mapping. Gel slices containing gp100 and gp46 were excised from a SDS gel and digested with different amounts of V8 protease to obtain partial digestion patterns. Almost all peptides derived from gp46 comigrated with peptides from gp100 (Fig. 1B), suggesting that gp46 has significant regions of identity with a portion of gp100. These findings suggest that gp46 and gp100 are distinct isoforms of VCAM-1.

The glycosylation of gp100 and gp46 was characterized with glycosidases. Both gp100 and gp46 were reduced in apparent mass after treatment with neuraminidase (Fig. 1C, lanes 2 and 6) and *N*-glycanase (Fig. 1C, lanes 4 and 8), but not after treatment with *O*-glycanase (Fig. 1C, lanes 3 and 7).

Biosynthesis studies were carried out to determine whether there was a precursor/product relationship between gp100 and gp46. PA6 cells were pulse-labeled with ^{35}S-methionine and cysteine and chased for varying periods of time and both the cell lysate and supernatant were analyzed for gp100 and gp46 by immunoprecipitation with MVCAM-A mAb (**Fig. 2**). Both gp100 and gp46 were produced as smaller precursors (masses of about 94 kDa and 40 kDa, respectively) and converted into mature gp100 and gp46 within 1 h. This conversion is probably due to carbohydrate processing because both gp100 and gp46 have *N*-linked car-

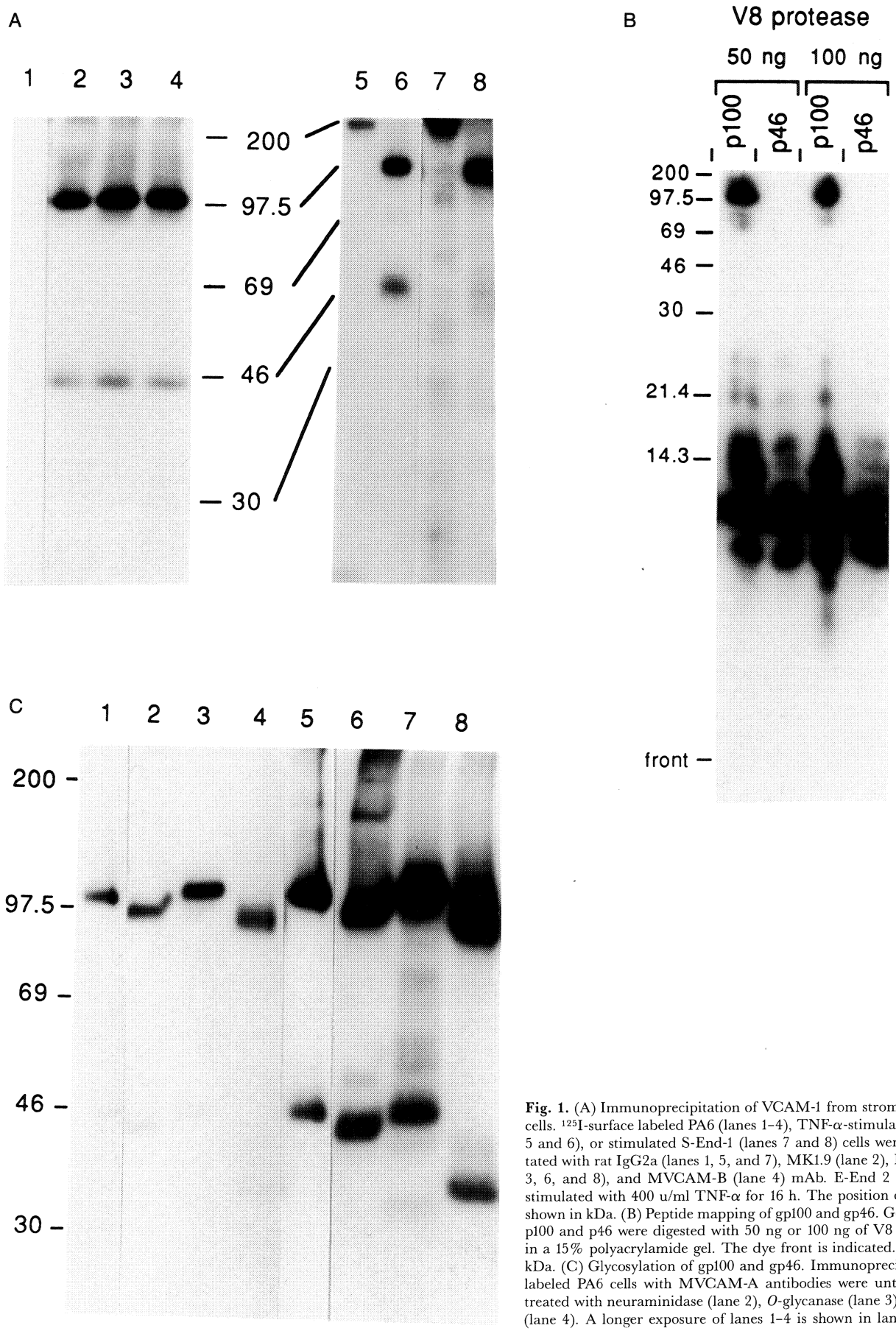

Fig. 1. (A) Immunoprecipitation of VCAM-1 from stromal and endothelial cells. ^{125}I-surface labeled PA6 (lanes 1–4), TNF-α-stimulated E-End 2 (lanes 5 and 6), or stimulated S-End-1 (lanes 7 and 8) cells were immunoprecipitated with rat IgG2a (lanes 1, 5, and 7), MK1.9 (lane 2), MVCAM-A (lanes 3, 6, and 8), and MVCAM-B (lane 4) mAb. E-End 2 and S-End-1 were stimulated with 400 u/ml TNF-α for 16 h. The position of mass markers is shown in kDa. (B) Peptide mapping of gp100 and gp46. Gel slices containing p100 and p46 were digested with 50 ng or 100 ng of V8 protease, and run in a 15% polyacrylamide gel. The dye front is indicated. Mass is shown in kDa. (C) Glycosylation of gp100 and gp46. Immunoprecipitates of surface-labeled PA6 cells with MVCAM-A antibodies were untreated (lane 1) or treated with neuraminidase (lane 2), *O*-glycanase (lane 3), and *N*-glycanase (lane 4). A longer exposure of lanes 1–4 is shown in lanes 5–8.

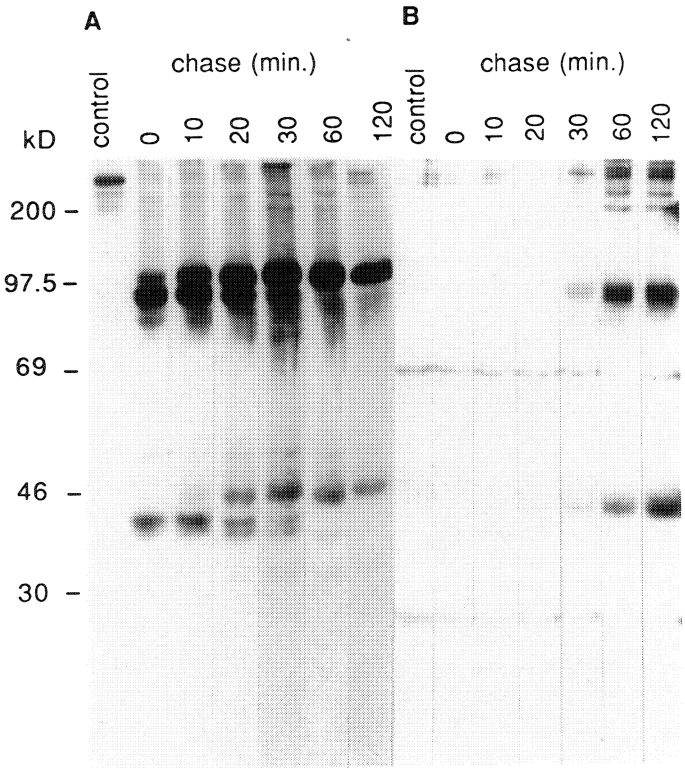

Fig. 2. Kinetics of biosynthesis of gp100 and gp46 and their shedding into the supernatant. PA6 cells were pulse-labeled with ^{35}S-methionine and cysteine and chased with an excess of methionine and cysteine, followed by immunoprecipitation of the entire cell lysate (A) and supernatant (B) with MVCAM-A antibodies (lanes 2–7) or control rat IgG2a (lane 1) and SDS-PAGE. Exposure times were 3 days for the cell lysates and 6 days for supernatants.

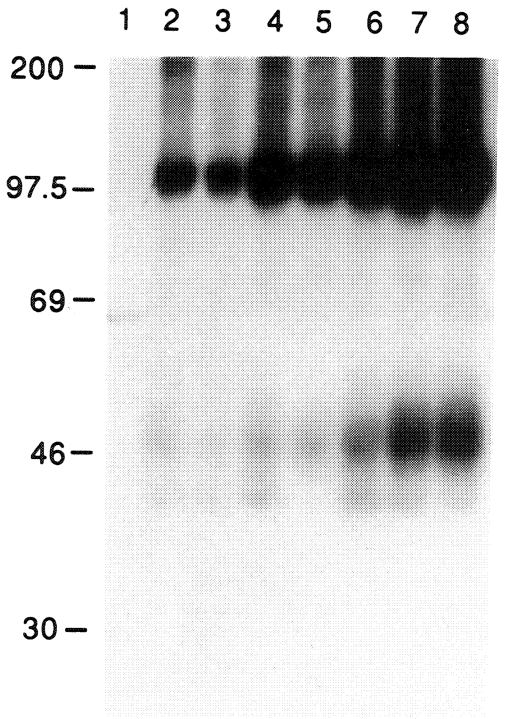

Fig. 3. Induction of gp46 and gp100. E-End-2 cells were unstimulated (lanes 1 and 2) or stimulated with TNF-α for 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 3 h (lane 6), 4 h (lane 7), and 5 h (lane 8), followed by cell-surface labelling with ^{125}I and immunoprecipitation with rat IgG2a (lane 1) or MVCAM-A (lanes 2–8).

bohydrate. Since gp100 and gp46 were synthesized with virtually identical kinetics, it is unlikely that gp46 is the product of proteolytic processing of gp100. Both gp100 and gp46 were shed into the supernatant beginning at 30 min (Fig. 2B; note the 2-fold longer exposure). The shed forms were slightly smaller than the cell-associated mature forms.

The kinetics of induction by TNF-α of surface expression of gp100 and gp46 was studied in E-End-2 cells (**Fig. 3**). Unstimulated E-End-2 cells expressed a small amount of VCAM-1 (Fig. 3, lane 2). Both gp100 and gp46 were increased by 1 h (Fig. 3, lane 4) and continued to increase for 5 h (Fig. 3, lanes 5–8).

To test gp46 and gp100 for GPI-anchoring, surface ^{125}I-radiolabeled cells were treated with PI-PLC followed by immunoprecipitation (**Fig. 4**). PI-PLC treatment of PA6 and TNF-stimulated E-End-2 cells resulted in a drastic decrease of gp46 in the cell lysate and a reciprocal increase in the supernatant, showing that gp46 is a GPI-linked cell surface molecule. The PI-PLC treatment did not cause any change in the amount of gp100 in the cell lysate and supernatant, confirming that gp100 has a polypeptide anchor. The amount of gp46 recovered in the supernatant after PI-PLC treatment was greater than that in the cell lysate without PI-PLC. This suggests that gp46 is not completely extracted from the cell membrane with lysis buffer containing Triton X-100. This phenomenon is well known for GPI-anchored proteins [28] and has previously been observed for the GPI-anchored isoform of LFA-3 [29]. We were not able to increase the extraction of gp46 using octyl glucoside (data not shown).

To examine whether the GPI-anchored gp46 isoform of VCAM-1 contributed to VLA-4 dependent adhesion, the ability of LyD9 cells to form conjugates with PA6- and TNF-stimulated E-End-2 cells was analyzed with or without treatment with PI-PLC. Conjugate formation between LyD9 and PA6 cells was almost completely VLA-4-dependent (**Table 1**). Treatment of PA6 with PI-PLC had no significant effect

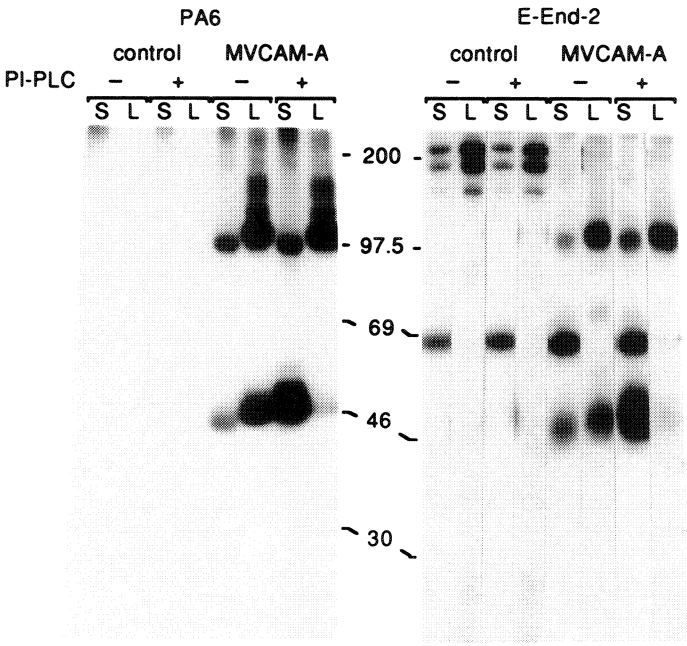

Fig. 4. Cleavage of gp46 with PI-PLC. Cell-surface ^{125}I-labeled PA6 and stimulated E-End-2 were incubated with (+) or without (–) PI-PLC for 1 h before supernatants (S) or lysates (L) were subjected to immunoprecipitation with rat IgG2a (control) or MVCAM-A.

on binding to LyD9, consistent with little release of VCAM-1 measured by immunofluorescent flow cytometry (data not shown) and quantitation of the relative amounts of the two isoforms with internal cysteine and methionine labeling as shown in Figure 2. In three different experiments with TNF-stimulated E-End-2 cells, conjugate formation was 40–46% VLA-4-dependent. Since conjugate formation was less dependent on VLA-4 than with PA6 cells, mAb to LFA-1 was also included in the experiments; however, baseline conjugate formation was only decreased 1 to 3% by LFA-1 mAb. PI-PLC treatment decreased conjugation with E-End-2 cells by a mean of 25%. PI-PLC treatment had no effect on conjugation in the presence of PS/2 mAb, suggesting that PI-PLC only affected VLA-4-dependent conjugate formation. Thus about half of the VLA-4-dependent conjugate formation was abolished by PI-PLC.

DISCUSSION

We studied two isoforms of VCAM-1, gp100 and gp46. The gp100 isoform has molecular properties equivalent to those previously described for mouse VCAM-1 [3]. Its size and polypeptide anchor correspond to a cDNA encoding 7 Ig-like domains and transmembrane and cytoplasmic domains [17]. The gp46 isoform is closely related to gp100, as shown by peptide mapping and cross-reaction with 3 mAbs that recognize gp100. gp46, and not gp100, is GPI-anchored, as shown by its release with PI-PLC. Both isoforms are independently biosynthesized and kinetics of processing and induction by inflammatory stimuli are similar. While this work was in progress, two groups reported a cDNA that encodes a GPI-anchored isoform of VCAM-1 [30, 31]. Compared to the cDNA encoding the transmembrane form of VCAM-1, this cDNA shares Ig-like domains 1–3, lacks Ig-like domains 4–7, and has a unique C-terminal hydrophobic domain that contains a signal for reanchoring to GPI. Transfection of COS cells demonstrated GPI anchoring, and binding to VLA-4 [30, 31]. The molecule expressed in COS cells has characteristics identical to that of gp46 studied here. Furthermore, the mAb M/K-2, which reacts with the cDNA expressed in COS cells, and the mAb M/K-1.9, shown here to recognize gp46, are two mAbs to VCAM-1 that have been shown to cross-block each another [3]. Work by the groups of Moy et al. [31] and Terry et al. [30] has focused on the cDNA, expression of the mRNA, and studies on the GPI-anchored isoform of VCAM-1 expressed in COS cells. However, one group demonstrated by immunoprecipitation that the GPI-anchored isoform was expressed in an SV40-transformed endothelial cell line [30]. Our work and a more recently published report [32] extend these previous studies by characterizing biosynthesis and by demonstrating that the protein product, and not only as previously reported the mRNA for the GPI-anchored isoform, is induced by inflammatory stimuli. Furthermore, we found that the protein products for the two isoforms are differentially expressed in cell lines. Both isoforms are basally expressed in the bone marrow-derived stromal cell line PA6, whereas TNF induced both isoforms in E-End-2 but only the gp100 isoform in S-End-1 endothelioma cell lines. Thus, the GPI isoform can be regulated differentially from the transmembrane isoform in cell lines. We further extend previous reports by demonstrating that on a physiological cell type, on which both isoforms are expressed, the GPI-anchored isoform is expressed in sufficient quantities to contribute to cell-cell adhesion.

In humans, alternatively spliced isoforms with six or seven Ig-like domains are found [1, 10, 12–14], but the isoform with six Ig-like domains is found only in trace amounts and is not known to have a different pattern of expression than the seven-domain isoform. It is interesting that in the mouse, the second binding site for VLA-4 in domain 4 can also be alternatively spliced out, but by a different mechanism resulting in loss also of domains 5–7 and use of a different membrane anchor [31]. We have provided evidence in the mouse for differential expression of the two isoforms, suggesting regulation of alternative splicing. TNF induces both isoforms in E-End-2 endothelioma cells, but only gp100 in S-End-1 endothelioma cells. Quantitation of radioactivity in gp46 and gp100 in the pulse-chase experiments, using a phosphorimager and correction for the cysteine and methionine content predicted by the cDNAs for the two isoforms [17, 31], shows that PA6 stromal cells express 18.5 ± 2% as much gp46 as gp100. A caveat is that twice as much gp46 is released by PI-PLC as is solubilized by detergent and thus this may be an underestimate. The ratio of gp46 to gp100 in the ST2 stromal cell line was similar to that in PA6 (data not shown). Similar results were obtained in independent internal ^{35}S labelling experiments, whereas after ^{125}I external labeling, the ratio of the two isoforms varied between experiments (compare PA6 cells in Fig. 1A and Fig. 4).

Our functional studies suggest that gp46 can contribute to VLA-4-dependent adhesion, even though expressed in lower amounts than gp100. This contribution was apparent on E-End-2 cells, the strongest expressor of gp46 relative to gp100 studied here, but not on PA6 cells. PI-PLC reduced adhesion of E-End-2 cells to LyD9 and this reduction was not additive with inhibition by VLA-4 mAb, suggesting that removal of a VLA-4 ligand, most likely gp46, was responsible for the inhibition by PI-PLC. Inhibition by PI-PLC was 59% of the inhibition by VLA-4 mAb, suggesting a substantial contribution by gp46 to interaction with VLA-4. By contrast, cells that lack the GPI-anchored isoform of LFA-3 are as efficient in CD2-dependent functions as cells with both GPI- and transmembrane-anchored isoforms [33]. The GPI-anchored

TABLE 1. Effect of PI-PLC Treatment on Conjugation of LyD9 Cells with PA6 or TNF-stimulated E-End-2 cells

		PA6	E-End-2[b]		
			Exp. 1	Exp. 2	Exp. 2
VLA-4 mAb[a]	PI-PLC	% conjugates		% conjugates	
–	–	37.0	27.8	26.6	36.9
–	+	38.5	18.9	19.0	31.5
+	–	7.2	15.5	15.1	22.1
+	+	7.4	N.D.	15.5	25.1

[a]VLA-4 mAb PS/2 was used at 20 μg/ml. For PA6 cells, MVCAM-B was used with PS/2 at 20 μg/ml. Similar results were obtained in multiple experiments with PA6 cells.

[b]For E-End-2 experiments, M17/4 mAb to LFA-1 was included in all experimental groups, which decreased conjugates by 1 to 3% in the three different experiments.

isoform of VCAM-1 might have greater mobility in the cell membrane [34] and hence be able to interact more efficiently with VLA-4. Further studies are required to define whether the two isoforms of VCAM-1 differ in membrane mobility, topographic distribution in the membrane, signaling, or affinity for VLA-4 and thus have differential biological functions.

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