

# Activation-dependent Recognition by Hematopoietic Cells of the LDV Sequence in the V Region of Fibronectin

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**Abstract.** It has been shown that the  $\alpha 4\beta 1$  integrin is the lymphocyte receptor for the carboxy terminal cell-binding domain of fibronectin which comprises adhesion sites in Hep 2 and a high affinity site, CS-1, in the type III connecting segment or V (for variable) region. In the present studies, using a series of peptides derived from CS-1, we identify the tripeptide leu-asp-val (LDV), as the minimal peptide capable of supporting stable lymphocyte or melanoma cell adhesion. However, only cells which expressed an active form of the  $\alpha 4\beta 1$  complex were capable of attaching to and spreading on LDV peptide. On a molar basis, LDV minimal peptides were either not active or 10–20 times less active than intact CS-1 in promoting the adhesion of lymphocytes expressing the resting form of the receptor. In cells which express the high avidity form of the receptor, LDV and CS-1 were equally effective in promoting cell adhesion and spreading.

The avidity of the  $\alpha 4\beta 1$  complex could be altered with mAbs to  $\beta 1$  which specifically activate  $\beta 1$  dependent function. The high avidity form of the  $\alpha 4\beta 1$  complex could be induced on U937 cells, T, and B lymphoblastoid cell lines, or PHA-stimulated T cell blasts. Resting PBL could not be induced to bind LDV peptide conjugates by activating antibodies to  $\beta 1$  implying that two signals are required for LDV recognition by T cells. In conclusion, these data show clearly that the minimal peptide for the  $\alpha 4\beta 1$  complex in CS-1 is the LDV sequence. Although numerous cell populations can interact with intact CS-1 only cells which express an active  $\alpha 4\beta 1$  complex can bind the LDV sequence. This implies that cell interaction with the carboxy terminal cell-binding domain of fibronectin can be regulated at several levels: (a)  $\alpha 4\beta 1$  expression; (b) activation of the  $\alpha 4\beta 1$  complex; and (c) alternate splicing of CS-1 into V+ isoforms of fibronectin.

VARIOUS cell populations can interact with adhesion sequences in the carboxy terminal cell-binding domain (CTCBD)<sup>1</sup> of fibronectin (McCarthy et al., 1986; Bernardi et al., 1987; Humphries et al., 1986, 1987; Liao et al., 1989; Mould et al., 1990; Wayner et al., 1989; Garcia-Pardo et al., 1990; Guan and Hynes, 1990), in addition to the arg-gly-asp-ser (RGDS) adhesion sequence located in the central cell binding domain (CCBD) of fibronectin (Pierschbacher and Ruoslahti, 1983). Lymphocytes, in particular, bind with high affinity to a 38-kD tryptic fragment of fibronectin (Garcia-Pardo et al., 1987; Garcia-Pardo et al., 1990; Wayner et al., 1989) which contains the Heparin 2 (Hep 2) domain and 67 (out of 120) amino acid residues of the alternatively spliced type III connecting segment or V (for variable) region (Schwarzbauer et al., 1983, 1985; Kornblihtt et al., 1985; Paul et al., 1986). The 67 amino acids of this fragment (Garcia-Pardo et al., 1987) which are

derived from alternative splicing of the fibronectin gene span the CS-1, CS-2, and CS-3 regions defined by Humphries et al. (1986, 1987). Our previous findings indicated that it was the CS-1 sequence which contributed to the high affinity binding site for lymphocytes on this fragment (Wayner et al., 1989; Garcia-Pardo et al., 1990). Interestingly, in human fibronectin two splice sites exist in V120 cDNA (Kornblihtt et al., 1985; Paul et al., 1986), one at the COOH-terminal end of CS-1 and another just NH<sub>2</sub>-terminal to the CS-5 region. Therefore, the entire CS-1 region can either be present or absent in V+ fibronectins. The importance of this with regard to lymphocyte function is, at present, unknown.

mAb inhibition studies showed that it was the  $\alpha 4\beta 1$  integrin receptor which mediated lymphocyte binding to CS-1 (Wayner et al., 1989; Garcia-Pardo et al., 1990). This finding was later confirmed by affinity chromatographic isolation of the  $\alpha 4\beta 1$  integrin from hematopoietic and melanoma cell populations on CS-1 sepharose (Mould et al., 1990; Guan and Hynes, 1990). Further, it has been reported that melanoma cells can recognize and bind the LDV sequence located in the COOH-terminal portion of CS-1 strongly suggesting that LDV is a peptide ligand for  $\alpha 4\beta 1$  (Mould et al., 1991). However, the minimal sequence in CS-1 capable of

Requests for the 8A2 mAb should be directed to Dr. N. Kovach.

1. *Abbreviations used in this paper:* CCBD, central cell binding domain; CTCBD, carboxy terminal cell-binding domain; Hep2, Heparin 2; LDV, leu-asp-val; RSA, rabbit serum albumin.

promoting stable hematopoietic cell adhesion has not yet been identified although two recent studies have shown that hematopoietic cells can interact with LDV-containing peptides derived from the COOH-terminal portion of CS-1 (Garcia-Pardo et al., 1990; Guan and Hynes, 1990).

In the present paper we have identified the minimal peptide ligand in CS-1 that is capable of supporting stable hematopoietic cell adhesion via  $\alpha 4\beta 1$ . Surprisingly, this varied according to the cell population examined. Although the minimal peptide for melanoma cell adhesion was leu-asp-val (or LDV), many hematopoietic cell lines required larger portions of the COOH-terminal end of CS-1, while still other populations could be identified that required the entire length of CS-1 to form stable attachments. This suggested that the LDV sequence might be recognized by some cell populations only in the context of intact CS-1 and that this recognition may be regulated in a cell type-specific manner. Further studies revealed that LDV recognition was in fact determined by the avidity of the  $\alpha 4\beta 1$  complex expressed by an individual cell population. The low avidity receptor, expressed on Jurkat, Ramos, U937, or PHA-activated T cells, could not bind LDV outside the context of CS-1 while the high avidity receptor, expressed by HUT 78 or A375 melanoma cells, could directly interact with LDV peptide-coated surfaces. The avidity of the  $\alpha 4\beta 1$  complex could be altered by a mAb to  $\beta 1$ , 8A2, which up-regulates  $\alpha 4\beta 1$ -dependent function (Kovach et al., 1992) but not surface expression (this manuscript). Therefore, in the presence of mAb 8A2 hematopoietic cells could be induced to form stable attachments to LDV-coated surfaces. This suggests that recognition of the LDV sequence in CS-1 requires activation of the  $\alpha 4\beta 1$  complex. Finally, PHA-stimulated but not resting T cells could be induced by mAb 8A2 to bind LDV suggesting that resting T cells require an additional signal(s) for LDV recognition.

Together, these data strongly suggest that hematopoietic cell interaction with the CTCBD of fibronectin is regulated at several levels: (a)  $\alpha 4\beta 1$  expression; (b) activation of  $\alpha 4\beta 1$ ; (c) presence of the CS-1 sequence in V+ fibronectin isoforms. Furthermore, these data also suggest that multiple signals, one of which is transduced through  $\beta 1$ , are required for T lymphocyte activation and LDV recognition.

## Materials and Methods

### Materials

Fibronectin was purified from human plasma as previously described (Wayner and Carter, 1987). Fragments of fibronectin were the same as previously described (Wayner et al., 1989; Garcia-Pardo et al., 1987).  $^{51}\text{Cr}$ -sodium chromate was from New England Nuclear (Boston, MA). Rabbit serum albumin (RSA), BSA and protein A-agarose were from Sigma Chemical Co. (St. Louis, MO). Protein G-agarose was a generous gift from Dr. Kurt Gehlsen (Pharmacia, La Jolla, CA).

### Peptides and Peptide Conjugates

A series of peptides spanning the entire CS-1 region were synthesized by Drs. Jim Blake and Wes Cosand (Oncogen, Seattle, WA) using an Applied Biosystems 430A peptide synthesizer and were a generous gift from the Bristol Myers-Squibb Pharmaceutical Research Institute, Oncogen Division (Seattle, WA). Synthetic peptides were HPLC purified and tested for direct toxicity and growth-inhibitory activity. Some peptides were synthesized with an  $\text{NH}_2$ -terminal cysteine at the end of a gly-gly-gly tail and were chemically conjugated to SMCC-derivatized RSA for use in cellular adhesion assays. None of the peptides, either inhibitory or noninhibitory, or any of the peptide conjugates were toxic or growth inhibitory.

## Cells and Cell Culture

The A375 (human melanoma) cell line was obtained from Dr. Diane Horn (Oncogen, Seattle, WA) and the Jurkat (human T lymphoblastoid) cell line was obtained from Dr. Dave Urdal (Immunex Corp, Seattle, WA). The HT1080, RD, Ramos, and HUT 78 cells were obtained from the American Type Culture Collection (Rockville, MD). All cell culture conditions were as previously described (Wayner and Carter, 1987). PHA-stimulated T cell blasts were prepared exactly as described from normal fresh human blood (Wayner et al., 1989).

## Monoclonal Antibodies

All mAbs to adhesion receptors were produced and characterized as described (Wayner and Carter, 1987; Wayner et al., 1989; Kovach et al., 1992). The 8A2 mAb has been shown to recognize an epitope on the integrin  $\beta 1$  subunit using the previously described anti- $\beta 1$ , P4C10 (Kovach et al., 1992). The anti- $\alpha 4$  mAb P4C2 has been previously described (Wayner et al., 1989). In every case, control antibody consisted of protein G-purified nonimmune mouse IgG.

### Activation of $\beta 1$ with Mab 8A2

The  $\beta 1$  subunit was activated with mAb 8A2 several ways. In some experiments, cell adhesion assays were carried out in the presence of 8A2. In other experiments, cells were pretreated with 8A2 mAb for 30 min, washed, and then used in cell adhesion assays. The effects of 8A2 mAb could be measured within 10 min and as little as 0.1  $\mu\text{g}/\text{ml}$  8A2 could stimulate  $\beta 1$  function.

### Inhibition of Cell Adhesion to Intact Fibronectin and CS-1 Peptide-RSA Conjugates with Monoclonal Antibodies

Antibodies that would alter cell adhesion to fibronectin or CS-1 peptide RSA conjugates were identified as previously described (Wayner and Carter, 1987; Wayner et al., 1989). Briefly, 48-well virgin styrene plates (No. 3547; Costar, Cambridge, MA) were coated with 5  $\mu\text{g}/\text{ml}$  plasma fibronectin, or peptide conjugate (with the final concentration of peptide being 5  $\mu\text{g}/\text{ml}$ ).  $\text{Na}_2^{51}\text{CrO}_4$ -labeled cells were incubated with mAbs to adhesion receptors for 15 min at room temperature and were then allowed to attach to the coated substrates in the presence of the antibodies for 30–60 min at 37°C. In some cases antibodies to adhesive ligands were pre-incubated with the substrates for 15 min before the cells were added. At the end of the incubation, nonadherent cells were removed by washing with PBS and the adherent cells were dissolved in 0.1 N NaOH/0.25% SDS and bound  $^{51}\text{Cr}$  cpm were quantitated in a gamma counter.

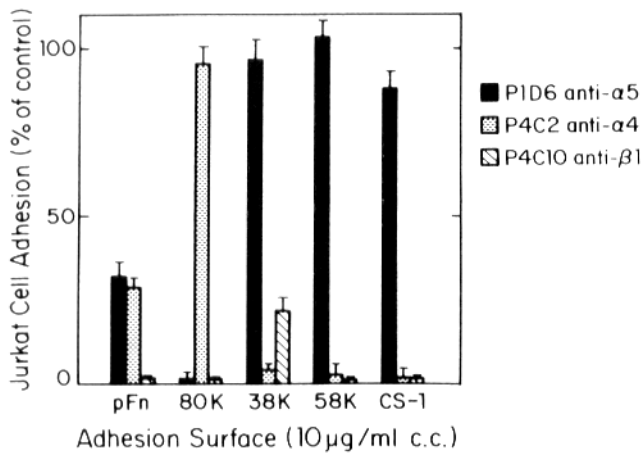
### Inhibition of Cell Adhesion to Fibronectin with CS-1 Derived Peptides

For peptide inhibition studies,  $^{51}\text{Cr}$ -labeled cells were pre-incubated with CS-1-derived peptides at various concentrations for 15 min at room temperature. The cells were then allowed to attach to fibronectin-coated surfaces in the presence of exogenous peptides for 30–60 min. The assay then proceeded as described above.

## Results

### $\alpha 4\beta 1$ Is the Receptor for the CS-1 Sequence Located in the Carboxy Terminal Cell-binding Domain of Fibronectin

We and others have previously reported that antibodies specific for epitopes on the  $\alpha 4$  subunit inhibit T or B lymphocyte adhesion to plasma fibronectin, fragments of fibronectin containing adhesion sites in the Hep 2 domain, and the first 25 amino acids (CS-1) of the V (for variable) region (Wayner et al., 1989; Garcia-Pardo et al., 1990; Guan and Hynes, 1990). Other workers have shown that the conjugate of  $\alpha 4$  with  $\beta 1$  can be affinity purified on immobilized CS-1



**Figure 1.** Adhesion of Jurkat T lymphoblastoid cells to plasma fibronectin, fragments of plasma fibronectin, or CS-1-rsa peptide-coated surfaces in the presence of inhibitory anti-integrin mAbs. <sup>51</sup>Cr-labeled cells were incubated in the presence of the indicated mAbs (10 µg/ml-purified antibody) for 10 min at ambient temperature and allowed to attach to the protein or peptide coated-surfaces for 15–30 min in the presence of the inhibitory mAbs. Adhesion is expressed as percent of control (protein G-purified non-immune mouse IgG). The plasma fibronectin and the plasma fibronectin fragments are identical to those used and described previously (Wayner et al., 1989).

(Mould et al., 1990; Guan and Hynes, 1990) indicating that the cellular receptor for CS-1 is  $\alpha 4\beta 1$ . However, the contribution of  $\beta 1$  in mediating adhesion to fragments of fibronectin containing Hep 2 (58 kD derived from the B chain of plasma fibronectin) or the high affinity CTCBD (38 kD derived from the A chain of fibronectin and contains Hep 2 and CS-1) has not yet been elucidated. Therefore, we examined the adhesion of T (Jurkat; Fig. 1) or B (not shown) lymphoblastoid cell lines to surfaces coated with various fragments of fibronectin in the presence of mAbs to inhibitory epitopes on  $\alpha 4$  or  $\beta 1$ . The data in Fig. 1 show clearly that Jurkat cell adhesion to plasma fibronectin, CS-1, or fragments of fibronectin containing Hep 2 (58 kD) and the entire CTCBD (38 kD) can be inhibited by mAbs to  $\alpha 4$  (P4C2) or  $\beta 1$  (P4C10). This confirms the role of  $\beta 1$  in mediating adhesion of cells to the Hep 2 site as well as to CS-1. As we have already reported, adhesion to the CCBd (80-kD fragment) which contains the RGDS sequence is inhibited by mAbs directed to  $\alpha 5$  (PID6) or  $\beta 1$  (P4C10).

#### Identification of the Minimal Peptide Ligand for the Lymphocyte $\alpha 4\beta 1$ Receptor in CS-1

The first step taken to define a minimal peptide ligand in CS-1 for the  $\alpha 4\beta 1$  integrin receptor was to divide CS-1 into two smaller peptides, an NH<sub>2</sub>-terminal (A13) and a COOH-terminal (B12) peptide. The ability of these and smaller peptides to inhibit Jurkat cell adhesion to substrates coated with intact fibronectin was examined (Table I). As we and others (Humphries et al., 1986, 1987; Garcia-Pardo et al., 1990; Guan and Hynes, 1990) have reported CS-1 was a potent inhibitor of cell adhesion to fibronectin (Table I). Interestingly, only the COOH-terminal B12 peptide was effective in inhibiting T cell adhesion to intact fibronectin (Table I). This is in agreement with our previously published findings with

**Table I. Molar Concentration of CS-1 Peptides Required for 50% Inhibition of Jurkat Cell Adhesion to Plasma Fibronectin**

Peptide	Sequence	Molar concentration (nM)
CS-1	DELPLVTLPHPNLHGPEILDVPST	0.18
B12	LHGPEILDVPST	0.40
	GPEILDVPST	0.45
	EILDVPST	0.55
	LDVPST	>1.60
	VPST	>2.50
	EILDV	0.66
	LDV	>2.70
A13	DELPLVTLPHPN	>1.00

<sup>51</sup>Cr-labeled Jurkat cells (10<sup>5</sup>/well) were incubated in the presence of the varying concentrations (starting at 2 mg/ml) of the indicated peptides for 15 min. The Jurkat cells were then allowed to adhere to plasma fibronectin-coated surfaces (5 µg/ml) in the presence of the peptides for 30 min. Adhesion to fibronectin was evaluated as cpm bound to the fibronectin surface (Y axis), plotted as a function of molar peptide concentration (X axis) and the 50% inhibition point was determined.

human B lymphocytes (Garcia-Pardo et al., 1990) and also corroborates another report identifying the GPEILDVPST as the active peptide for murine hematopoietic cell adhesion (Guan and Hynes, 1990). Next, a series of smaller peptides derived from B12 were tested for their ability to inhibit Jurkat cell adhesion to intact fibronectin (Table I). The results show clearly that deletion of the NH<sub>2</sub>-terminal LHGP or COOH-terminal PST residues had little effect on the ability of a particular B12 derived peptide to inhibit Jurkat adhesion to fibronectin. In fact, several of these truncated B12 peptides (Table I) were similar in their ability to inhibit T cell fibronectin interaction as long as the minimal EILDV sequence was conserved (Table I). Interestingly, although LDV has been reported to be the minimal peptide for the melanoma  $\alpha 4\beta 1$  receptor (Mould et al., 1991; Komoriya et al., 1991), LDV was not capable of inhibiting Jurkat-fibronectin adhesion. The data in Table I suggest that the minimal peptide ligand in CS-1 for the T lymphocyte  $\alpha 4\beta 1$  receptor is glu-iso-leu-asp-val or EILDV. Identical results were obtained with a B lymphoblastoid cell line, Ramos (not shown) and an identical pattern was observed for peptide inhibition of Jurkat or Ramos cell adhesion to CS-1-coated surfaces;

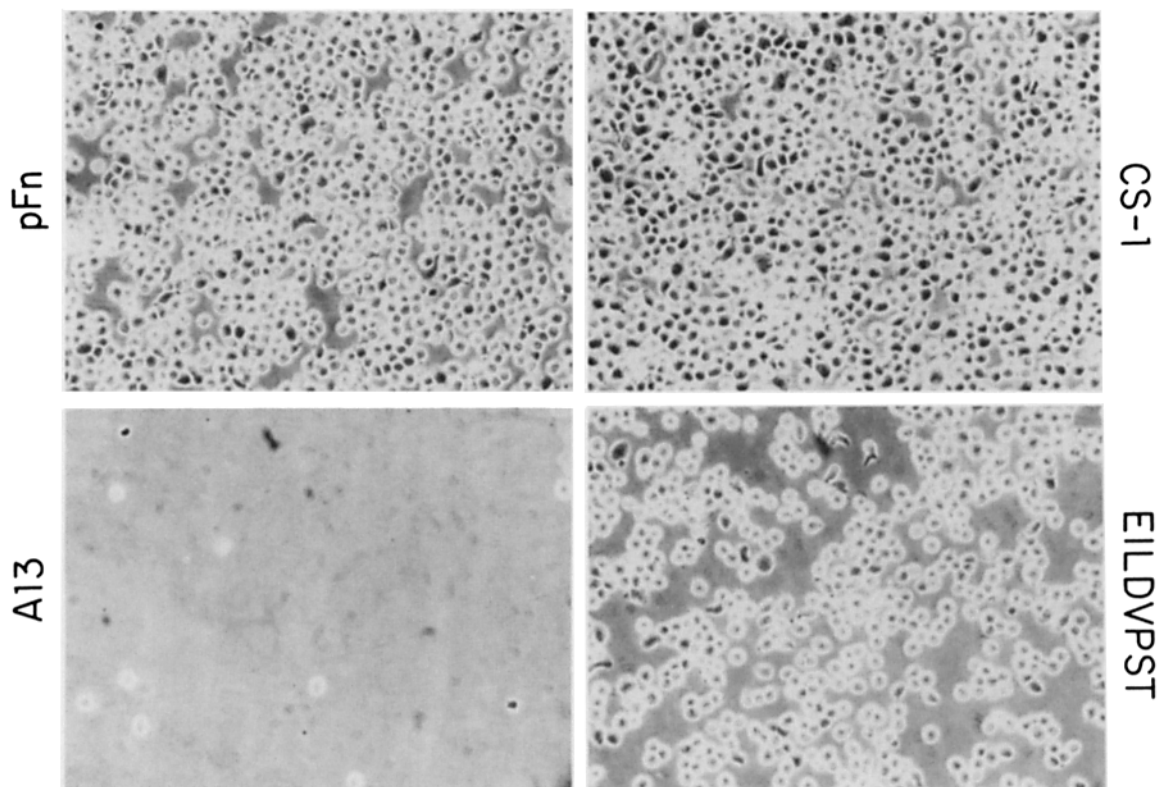
**Table II. Molar Concentration of CS-1 Peptide Conjugates Required for 50% Adhesion of Jurkat Cells**

Peptide or fragment	Molar concentration (nM)
rsa-CS-1	115.0
rsa-B12	492.0
rsa-EILDVPST	1,007.0
rsa-EILDV	1,129.0
rsa-LDV	>2,000.0
rsa-A13	No adhesion

<sup>51</sup>Cr-labeled Jurkat cells (10<sup>5</sup>/well) were incubated on surfaces coated with varying concentrations of peptide-rsa conjugates starting at 50 µg/ml (wt/vol) fragment or peptide (based on peptide not peptide-conjugate weight). Adhesion was evaluated as cpm bound to the surfaces (Y axis), plotted as a function of fragment or peptide concentration (X axis) and the molar concentration of a peptide required to support 50% (of total input cpm) adhesion was calculated. No adhesion, no measurable adhesion above negative controls.

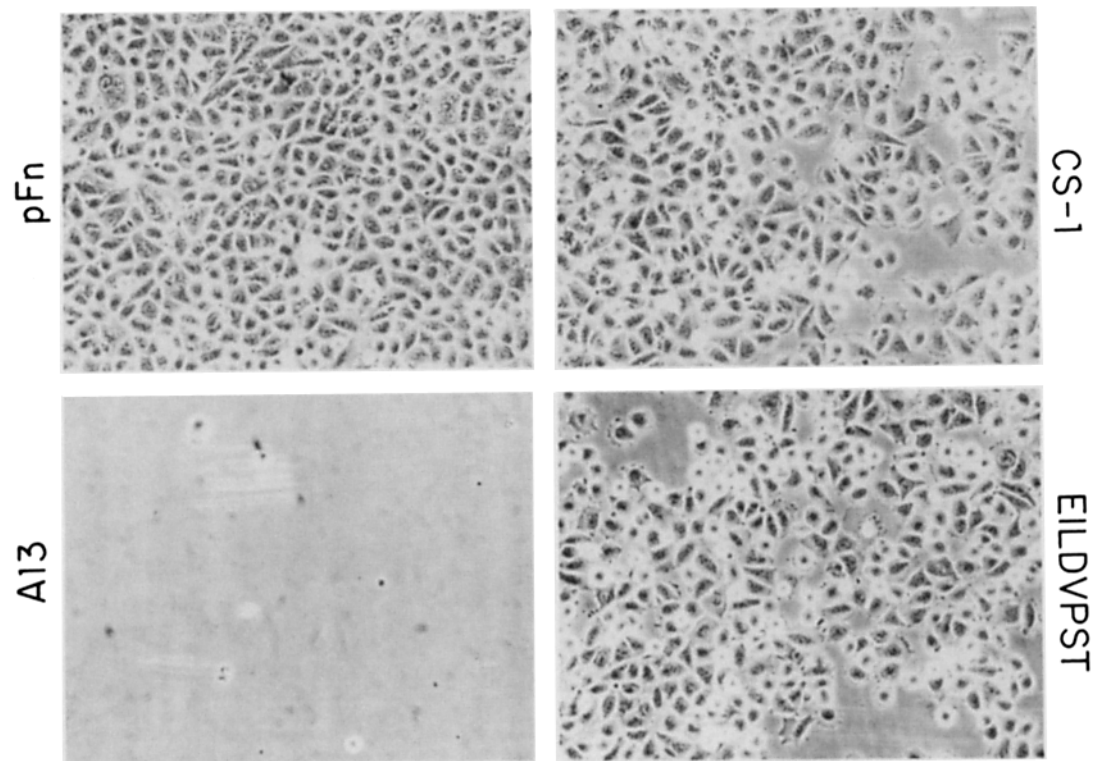
**A**

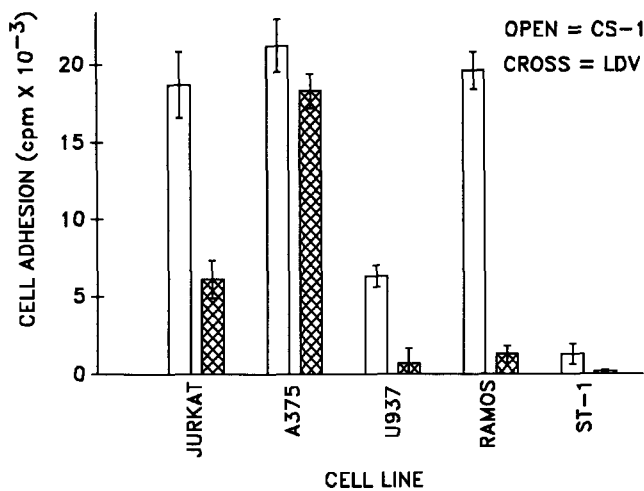
JURKAT



**B**

A375



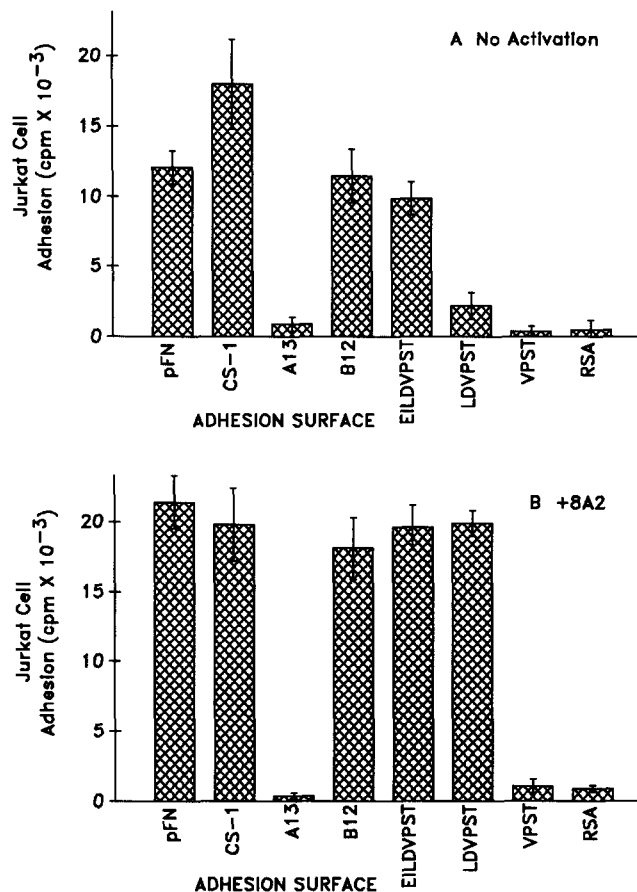


**Figure 3.** Adhesion of various hematopoietic cell lines to CS-1 (□) or LDV (▨)-coated surfaces. <sup>51</sup>Cr-labeled Jurkat (*T lymphoblastoid*), A375 (*melanoma*), U937 (*monocytic*), Ramos (*B lymphoblastoid*), and ST-1 (*EBV transformed B lymphoblastoid*) cells were allowed to adhere to CS-1-rsa or LDVPST-rsa (5 μg/ml peptide)-coated surfaces for 30 min at 37°C. At the end of this time the nonadherent cells were washed off and the adherent cells were solubilized in NaOH/SDS and quantitated in a gamma counter. The results are expressed as bound counts per minute.

deletion of the NH<sub>2</sub>-terminal glutamic acid and isoleucine residues resulted in a peptide with no inhibitory activity (not shown).

#### Minimal CS-1 Peptides Support Stable Cell Adhesion and Spreading

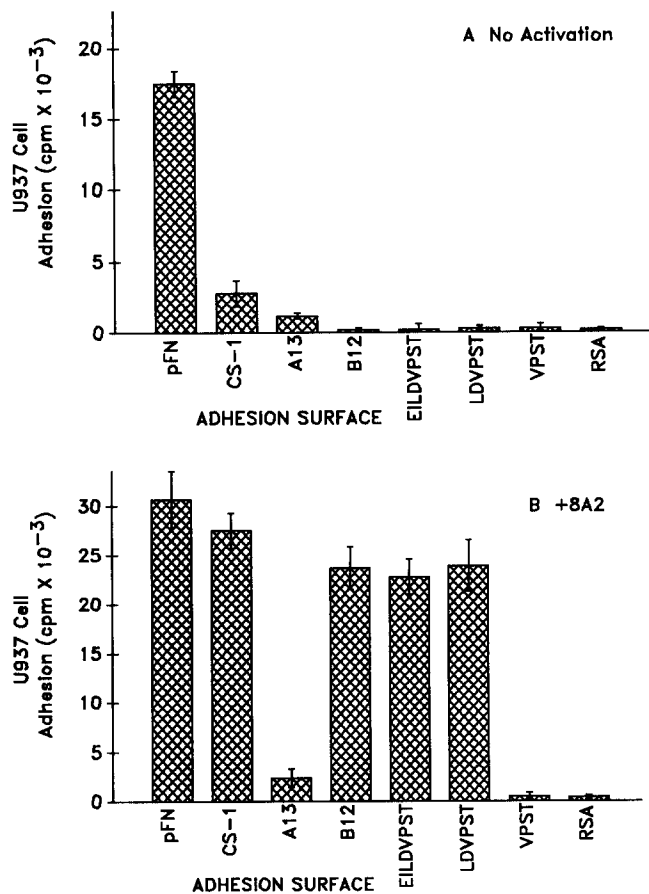
It was of interest to determine if CS-1 or derivative peptides were capable of inducing stable hematopoietic cell adhesion and spreading. Therefore, RSA-peptide conjugates were prepared and their ability to support cell adhesion was examined and compared to intact fibronectin and fragments of fibronectin containing the CTCBD (Table II and Figs. 2 and 3). As expected CS-1 and a peptide containing EILDV were able to support the adhesion of Jurkat and A375 melanoma cells (Fig. 2, A and B). Interestingly, peptide conjugates containing EILDV (Fig. 2 B) or LDV (not shown) also supported melanoma cell spreading. However, on a molar basis there were significant differences in the ability of CS-1 versus CS-1 derivative peptides to promote Jurkat cell adhesion (Table II). In general, truncated CS-1 peptides were inefficient mediators of hematopoietic cell adhesion and none of the truncated CS-1 peptide conjugates could support the adhesion of PHA activated T cell blasts (not shown). Furthermore, although the minimal peptide sequence required to support melanoma cell adhesion has been reported to be LDV (Mould et al., 1991; Komoriya et al., 1991) and, in our hands, A375 melanoma cells did adhere to LDVPST-coated surfaces (Fig. 3), of the hematopoietic cell lines we exam-



**Figure 4.** Adhesion of Jurkat cells to surfaces coated with pFN-, CS-1-, A13-, or B12-derived peptide-rsa conjugates in the presence of mAb 8A2. (A) Adhesion in the presence of purified nonimmune mouse IgG (5 μg/ml). (B) Adhesion in the presence of mAb 8A2 (5 μg/ml). 48-well plates were coated with 5 μg/ml (based on peptide weight) peptide-rsa conjugates overnight in PBS at 4°C. <sup>51</sup>Cr-labeled Jurkat cells were allowed to adhere to the peptide-coated surfaces in the presence of IgG or 8A2 for 30 min at 37°C. The rest of the adhesion assay proceeded as for Fig. 3. Results are expressed as bound counts per minute.

ined only Jurkat cells adhered slightly to surfaces coated with the LDVPST-rsa conjugate (Fig. 3). Furthermore, some of the hematopoietic cell lines we examined, such as U937 or ST-1 cells did not adhere to surfaces coated with intact CS-1 (Fig. 3). The reason for the apparent inability of some cell populations to adhere to CS-1 or LDVPST-coated surfaces was not immediately obvious. Flow cytometry analysis revealed that cell surface expression of α4 or β1 could not account for the functional differences we observed in the ability of a particular cell population to adhere to CS-1 or LDV-coated surfaces. As we have previously reported (Wayner et al., 1989) U937 (monocytic) and ST-1 (*B lymphoblastoid*) cells express high levels of cell surface α4 and β1

**Figure 2.** Adhesion of Jurkat (A) or A375 melanoma cells (B) to plasma fibronectin (pFN), CS-1, A13, or EILDVPST-coated surfaces. Plasma fibronectin (pFN) CS-1, A13, or EILDVPST-rsa conjugates were coated on virgin styrene surfaces (5 μg/ml). The A375 or Jurkat cells were allowed to adhere for 1 h. Nonadherent cells were washed off and the resulting monolayers were photographed with an inverting microscope and phase contrast. These data show that A375 cells spread on surfaces coated with LDV-containing peptides. Evidence of Jurkat spreading can also be seen with CS-1 (A, top right).

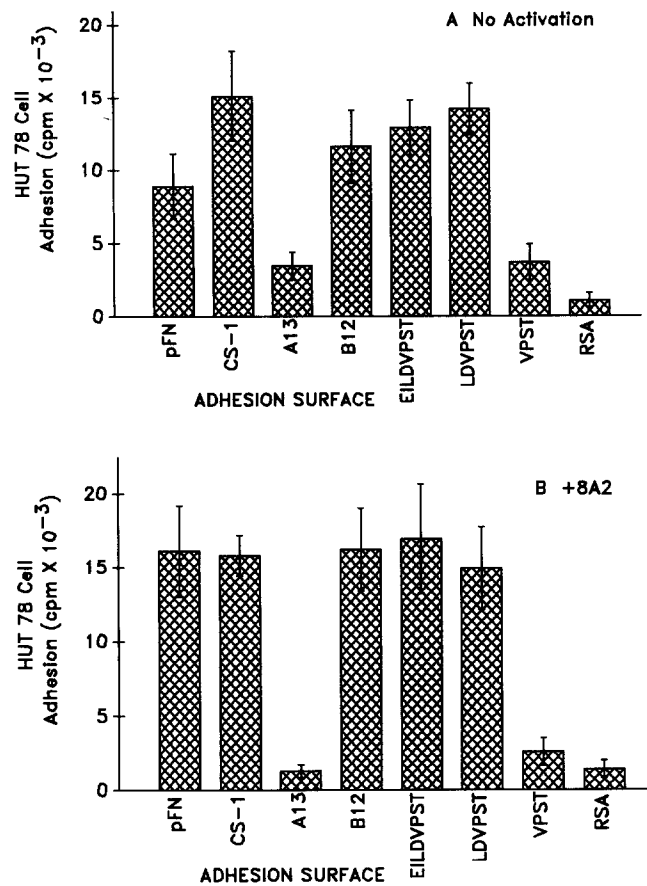


**Figure 5.** Adhesion of U937 cells to surfaces coated with pFN-, CS-1-, A13-, or B12-derived peptide-rsa conjugates in the presence of mAb 8A2. (A) Adhesion in the presence of purified nonimmune mouse IgG (5  $\mu$ g/ml). (B) Adhesion in the presence of mAb 8A2 (5  $\mu$ g/ml). 48-well plates were coated with 5  $\mu$ g/ml (based on peptide weight) peptide-rsa conjugates overnight in PBS at 4°C. <sup>51</sup>Cr-labeled U937 cells were allowed to adhere to the peptide-coated surfaces in the presence of IgG or 8A2 for 30 min at 37°C. The rest of the adhesion assay proceeded as for Fig. 3. Results are expressed as bound counts per minute. U937 cells do not adhere to B12 peptides without activation.

which are equivalent to the levels expressed by Jurkat and A375 melanoma cells (Wayner et al., 1989). Together, the data in Table II and Fig. 3 suggested that CS-1 or LDV-peptide recognition and binding might be regulated in a cell type specific manner with melanoma cells possessing the highest affinity receptor.

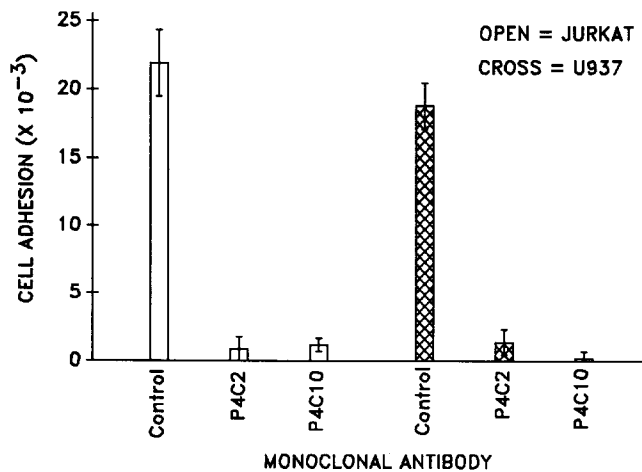
#### Activation of the $\alpha 4\beta 1$ Complex Enhances Hematopoietic Cell Recognition of CS-1 Peptides

The results of the preceding experiments suggested that adhesion of cells to CS-1 might involve the LDV sequence and that interaction of  $\alpha 4\beta 1$  with this sequence outside the context of intact CS-1 may be regulated in a cell-specific manner. Several reports have suggested that the interaction of cells with ECM proteins may require activation (Neugebauer and Reichardt, 1991; Shimizu et al., 1990). Furthermore, we have recently described a mAb to  $\beta 1$ , 8A2, that up-regulates  $\alpha 4\beta 1$ -dependent lymphocyte adhesion to VCAM-1



**Figure 6.** Adhesion of HUT 78 cells to surfaces coated with pFN-, CS-1-, A13-, or B12-derived peptide-rsa conjugates in the presence of mAb 8A2. (A) Adhesion in the presence of purified nonimmune mouse IgG (5  $\mu$ g/ml). (B) Adhesion in the presence of mAb 8A2 (5  $\mu$ g/ml). 48-well plates were coated with 5  $\mu$ g/ml (based on peptide weight) peptide-rsa conjugates overnight in PBS at 4°C. <sup>51</sup>Cr-labeled HUT 78 cells were allowed to adhere to the peptide-coated surfaces in the presence of IgG or 8A2 for 30 min at 37°C. The rest of the adhesion assay proceeded as for Fig. 3. Results are expressed as bound counts per minute. Unlike U937 cells, HUT 78 cells adhere to LDV-coated surfaces without activation.

(Kovach et al., 1991). Therefore, we examined the effects of 8A2 on hematopoietic cell adhesion to CS-1 and derivative peptides. These results are shown in Figs. 4 (Jurkat), 5 (U937 cells), and 6 (HUT 78). As can be seen from these data Jurkat or U937 cells are capable of maximal interaction with CS-1 and LDV-containing derivative peptides only after activation with the 8A2 mAb. U937 cells, in fact, do not adhere to any of the B12-derived peptides without activation. Since pretreatment of cells with 8A2 does not up-regulate expression of either  $\beta 1$  or  $\alpha 4$  on U937 cells (not shown), these data strongly suggest that recognition of the LDV sequence by  $\alpha 4\beta 1$  requires an activation signal which can be transduced through  $\beta 1$ . As we have previously shown (Tables I and II), VPST-rsa conjugates are inactive at inducing stable cell adhesion (Figs. 4, 5, and 6). These data strongly suggest that the minimal essential adhesion sequence in CS-1 for activated hematopoietic cells is LDV. Interestingly, HUT 78 cells appeared to possess an active  $\alpha 4\beta 1$  complex; resting HUT 78 cells adhered to LDV and this adhesion was not significantly up-regulated by 8A2 (Fig. 6).

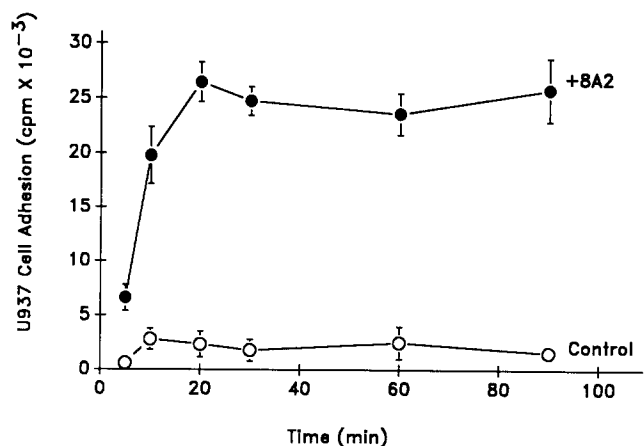


**Figure 7.** Adhesion of  $\beta 1$  activated Jurkat or U937 cells to LDVPST-coated surfaces in the presence of inhibitory monoclonal antibodies to  $\alpha 4$  (P4C2) or  $\beta 1$  (P4C10).  $^{51}\text{Cr}$ -labeled Jurkat ( $\square$ ) or U937 ( $\boxtimes$ ) cells were pre-incubated with 8A2 for 30 min at 37°C. At the end of this time, they were washed free of unbound 8A2, and incubated in the presence of nonimmune mouse IgG (10  $\mu\text{g}/\text{ml}$  = Control) or inhibitory mAbs to  $\beta 1$  (P4C10, 10  $\mu\text{g}/\text{ml}$ ) or  $\alpha 4$  (P4C2, 10  $\mu\text{g}/\text{ml}$ ) on the LDVPST-rsa-coated surfaces for 30 min at 37°C. Thus, the control as well as P4C10- or P4C2-treated cells were activated with 8A2.

The adhesion of 8A2 activated Jurkat or U937 cells to LDV peptide-coated surfaces could be completely abrogated by inhibitory mAbs to  $\alpha 4$  (P4C2) or  $\beta 1$  (P4C10) showing clearly that it is the conjugate of  $\alpha 4$  with  $\beta 1$  involved in LDV adhesion (Fig. 7). We have previously shown that P4C10 and 8A2 recognize functionally distinct epitopes on  $\beta 1$  (Kovach et al., 1991): 8A2 stimulates  $\beta 1$  function and P4C10 inhibits  $\beta 1$  function. Kinetic analysis of  $\beta 1$  activation by 8A2 revealed that 8A2 induces rapid and stable U937 (or Jurkat) cell adhesion to LDV peptide-coated surfaces. Within 5 min, significant adhesion can be measured which peaks at 10–20 min. In some cell populations, such as Jurkat cells (not shown) LDV recognition peaks within 5-min exposure to 8A2. Since resting U937 cells do not adhere to LDV-coated surfaces even after 90 min of incubation, these data clearly indicate that the effects of 8A2 are not simply on the rate of adhesion but are a function of altered LDV recognition presumably via an alteration in receptor conformation possibly resulting in increased receptor avidity and LDV recognition.

### Resting T Lymphocytes Require Two Signals for Adhesion to LDV

Interestingly, resting PBL did not adhere to CS-1 or LDV-coated surfaces even after pre-incubation with 8A2 (not shown). Peripheral blood-derived T lymphocytes required prior activation with PHA (72-h culture) to interact with LDV-coated surfaces in the presence of 8A2 (Fig. 9). As we have previously reported, PHA activation does increase  $\alpha 4\beta 1$  expression on T lymphocytes (Wayner et al., 1989). However, treatment with 8A2 does not increase surface expression of either  $\alpha 4$  or  $\beta 1$  on resting PBL or PHA-activated T cell blasts (not shown). Therefore, the effects of 8A2 on LDV recognition by PHA-activated T lymphocytes cannot be the result of increased  $\alpha 4\beta 1$  expression. This strongly sug-

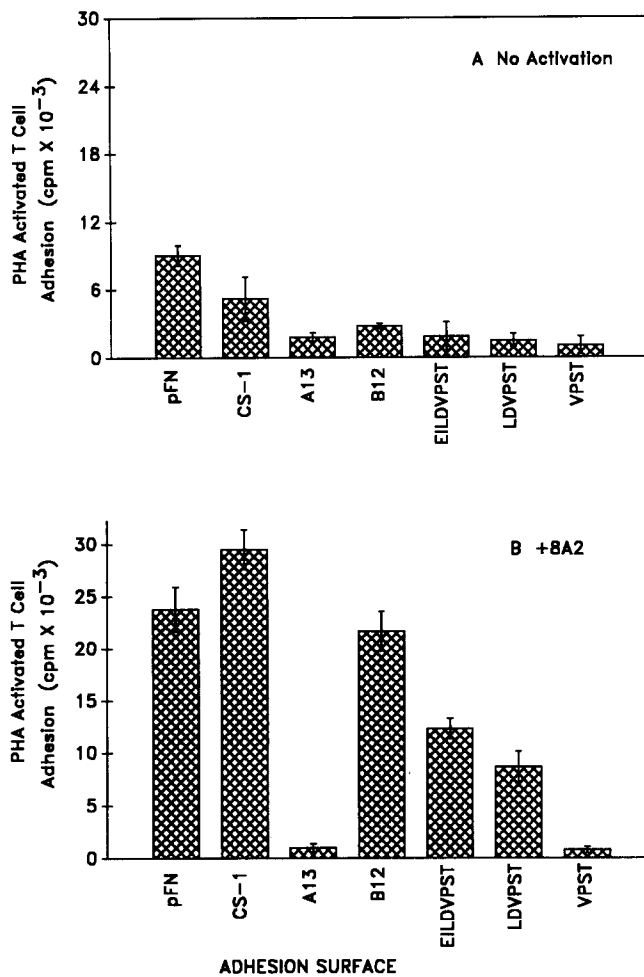


**Figure 8.** Kinetic analysis of U937 cell adhesion to LDV-peptide-coated surfaces in the presence of mAb 8A2.  $^{51}\text{Cr}$ -labeled U937 cells ( $2 \times 10^5$ ) were applied to triplicate wells of separate 48-well plates (one for each time point) which had been coated with 5  $\mu\text{g}/\text{ml}$  LDVPST-rsa. At time 0, 5  $\mu\text{g}/\text{ml}$  purified 8A2 was added to the wells designated as activated (control wells to which 5  $\mu\text{g}/\text{ml}$  nonimmune mouse IgG was added were included for each time point). Separate individual plates were harvested at each time point as described above. The results are expressed as bound counts per minute. In this experiment total cell input was  $28,796 \pm 1,389$ . Therefore, nearly maximal LDV-dependent adhesion was achieved after 5–10 min activation with 8A2.

gests that resting PBL, unlike cultured hematopoietic cell lines, require at least two signals for CS-1 (LDV) recognition, one of which is transduced through  $\beta 1$  (8A2) while the other is transduced through the T cell receptor (PHA).

### Discussion

Recently, it has been shown by several laboratories that lymphocyte integrin receptors (reviewed by Springer, 1990) can be activated to bind ligand with high avidity (Neugebauer and Reichardt, 1991; Kovach et al., 1991). Activation of integrins in T lymphocytes can be achieved by cross-linking the T cell receptor (Dustin and Springer, 1989; Shimizu et al., 1990) or by mAbs to  $\beta 1$  (Kovach et al., 1992). Therefore, we examined hematopoietic cell adhesion to CS-1 and derivative peptides in the presence or absence of an antibody known to activate  $\beta 1$  (Kovach et al., 1992). Hematopoietic cells which expressed the resting  $\alpha 4\beta 1$  complex did not recognize and bind LDV peptide. Such cell populations were only able to bind LDV in the context of CS-1 or in some cases CS-1-B12. However, after activating  $\beta 1$  with a mAb which up-regulates  $\beta 1$  function (Kovach et al., 1992) the minimal peptide ligand for the  $\alpha 4\beta 1$  receptor in hematopoietic cells was LDV. Furthermore, when the  $\beta 1$  complex was in the high avidity state, the molar concentration of LDV peptide required to support stable cell adhesion was similar to intact CS-1. We have previously shown that cross-linking of  $\beta 1$  by 8A2 is not required to up-regulate  $\beta 1$ -dependent function; Fab fragments are as effective as intact antibody (Kovack et al., 1992). Furthermore, we have also shown that 8A2 activation is energy dependent and does not result in the upregulation of surface expression of  $\alpha 4$  or  $\beta 1$ . Together these data strongly suggest that the adhesion of some cell populations to the LDV sequence in CS-1 is regulated in a cell-type



**Figure 9.** Adhesion of 72 h PHA-stimulated T cell blasts to pFN, CS-1, A13, or B12 and derivative peptide-coated surfaces. (A) Adhesion in the presence of purified nonimmune mouse IgG (5  $\mu$ g/ml). (B) Adhesion in the presence of mAb 8A2 (5  $\mu$ g/ml). 48-well plates were coated with 5  $\mu$ g/ml (based on peptide weight) peptide-rsa conjugates overnight in PBS at 4°C. <sup>51</sup>Cr-labeled PHA blasts were allowed to adhere to the peptide-coated surfaces in the presence of IgG or 8A2 for 30 min at 37°C. The rest of the adhesion assay proceeded as for Fig. 3. Results are expressed as bound counts per minute. Human PHA-stimulated T cell blasts were derived exactly as previously described (Wayner et al., 1989).

specific manner and requires an activation signal transduced through  $\beta$ 1.

Why some cells, such as Jurkat and Ramos, are able to recognize and adhere to CS-1-coated substrates but not LDV-coated substrates without  $\beta$ 1 activation is presently unclear. There are several possible explanations for this. First, there may be sequences in the NH<sub>2</sub>-terminal portion of CS-1 that function to alter the conformation of the LDV sequence to maximize a fit with the  $\alpha$ 4 $\beta$ 1-binding pocket. In this regard it is interesting that the CS-1 sequence contains 5 proline and 5 leucine residues. This combination of amino acids may result in folding of the CS-1 sequence so that the binding of LDV to  $\alpha$ 4 $\beta$ 1 is optimized. Since soluble A13 peptide does not enhance LDV recognition by Jurkat cells, this argues against a steric role for A13 in promoting LDV binding. In addition, since cells do not adhere to the A13 peptide even

after  $\beta$ 1 activation this argues against a direct role for this peptide in promoting cell adhesion.

A second possibility may be more likely. There may be sequences in the NH<sub>2</sub>-terminal portion of CS-1 which function to strengthen or "lock" the LDV peptide sequence into the  $\alpha$ 4 $\beta$ 1-binding pocket after the initial attachment event. This has been reported to occur for  $\alpha$ v $\beta$ 3 and the RGD sequence in vitronectin (Orlando and Cheresch, 1991). Precedence for this notion also comes from the work of Obara et al. (1988) in which a second site in the CCBD of fibronectin has been shown to act synergistically with the RGDS site in promoting cell adhesion and spreading. These "strengthening" or "synergistic" sequences in ligands may perform exactly the same function as the activating 8A2 antibody and alter receptor avidity by influencing the conformation of  $\beta$ 1. A model for this type of interaction can easily be envisioned. The ligand (in this case LDV)-binding pocket is created by a complex of  $\alpha$  and  $\beta$  (cations may also be involved). The LDV peptide binds to the receptor, this in turn may then immediately alter the conformation of the receptor such that the secondary "strengthening" or "synergy" site in  $\beta$ 1 engages to "lock" the receptor onto the ligand. It is this engagement which may define the high-avidity state. Cells such as HUT 78 and A375 melanoma may possess a permanently engaged receptor while Jurkat and Ramos cells possess a partially engaged receptor. This "locking" mechanism may also involve the transduction of a signal through  $\beta$ 1. The inhibitory anti- $\beta$ 1 mAb, P4C10, interferes with adhesion by inhibiting LDV binding. The activating anti- $\beta$ 1 mAb, 8A2, functions by mimicking the "strengthening" site. These distinct sites in  $\beta$ 1 act in concert to promote adhesion to any given ligand. Ligand specificity is therefore determined by  $\alpha$  and both sites in  $\beta$ 1 being engaged simultaneously. The concept of "binding" or "strengthening" sites being independently regulated provides an exquisitely dynamic system of checks and balances.

In conclusion, the present studies identify the LDV tripeptide as the minimal adhesive peptide in the CS-1 portion of the V region of fibronectin. However, only cells which express the high-avidity form of the receptor bind LDV coated surfaces. The high-avidity state of the receptor can be induced by preincubating cells with an activating mAb to  $\beta$ 1 (8A2). 8A2 activation of  $\beta$ 1 was rapid and did not result in an increase in cell surface  $\beta$ 1 expression. Resting peripheral blood T cells could not be induced by 8A2 to bind LDV while PHA stimulated T cell blasts could be. This implies that LDV recognition by normal T cells requires at least two signals, one of which is transduced through the T cell receptor (PHA activation) and the other which is transduced via the integrin  $\beta$ 1 subunit (8A2). Furthermore, since resting T cells, PHA blasts, HUT 78, and Jurkat cells do not demonstrate the same pattern and level of CS-1 peptide recognition, this suggests that malignant or activated T cells express receptor complexes that vary in terms of basal  $\alpha$ 4 $\beta$ 1 activation. The significance of this with regard to malignant T lymphocyte transformation and infiltration of organs such as the skin in neoplastic or chronic inflammatory disease has yet to be determined.

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