A Novel Integrin Specificity Exemplified by Binding of the $\alpha_v\beta_5$ Integrin to the Basic Domain of the HIV Tat Protein and Vitronectin

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Abstract. Several studies have addressed the interaction of the HIV Tat protein with the cell surface. Our analysis of the cell attachment-promoting activity of Tat and peptides derived from it revealed that the basic domain of Tat, not the arg-gly-asp (RGD) sequence, is required for cell attachment to Tat. Affinity chromatography with Tat peptides and immunoprecipitation with various anti-integrin antibodies suggest that the vitronectin-binding integrin, $\alpha_v\beta_5$, is the cell surface protein that binds to the basic domain of Tat. The Tat basic domain contains the sequence RKKRRQRRR. A related sequence, KKQRFRHRNRKG, present in the heparin-binding domain of an $\alpha_v\beta_5$ ligand, vitronectin, also bound $\alpha_v\beta_5$ in affinity chromatography and, in combination with an RGD peptide, was an inhibitor of cell attachment to vitronectin. The $\alpha_v\beta_5$ interaction with these peptides was not solely due to high content of basic amino acids in the ligand sequences; $\alpha_v\beta_5$ did not bind substantially to peptides consisting entirely of arginine or lysine, whereas a β_1 integrin did bind to these peptides. The interaction of $\alpha_v\beta_5$ with Tat is atypical for integrins in that the binding to Tat is divalent cation independent, whereas the binding of the same integrin to an RGD-containing peptide or to vitronectin requires divalent cations. These data define an auxiliary integrin binding specificity for basic amino acid sequences. These basic domain binding sites may function synergistically with the binding sites that recognize RGD or equivalent sequences.

THE tripeptide arg-gly-asp (RGD)[1] is required for cell adhesion to a number of proteins, including fibronectin, vitronectin, and fibrinogen (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1987). This adhesion is mediated by integrins, a family of transmembrane receptors composed of two subunits, α and β (Hemler, 1990; Ruoslahti, 1991; Hynes, 1992).

The Tat protein of human immunodeficiency virus (HIV-1) contains an RGD sequence and can mediate cell attachment in an RGD-dependent manner (Brake et al., 1990). Extracellular Tat is internalized by cells and transported to the nucleus, where it retains the ability to transactivate the HIV promoter (Frankel and Pabo, 1988). Furthermore, extracellular Tat has been shown to modulate cell proliferation, both in the suppression of proliferation of antigen-activated T-cells (Viscidi et al., 1989) and in the stimulation of proliferation of Kaposi's sarcoma (KS)-derived cells (Ensoli et al., 1990).

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1. *Abbreviations used in this paper*: RGD, arg-gly-asp; HIV, human immunodeficiency virus.

We felt that the possibility of an RGD-binding integrin mediating some of the interactions of Tat with cell surfaces was of a considerable interest and set out to identify such an integrin. We found that the $\alpha_v\beta_5$ integrin bound to the Tat protein, but that this interaction was not significant in the uptake of Tat by cells. Surprisingly, our results indicate that the binding of this integrin to Tat requires the basic region, whereas the RGD sequence is silent. We also provide evidence that a basic sequence in vitronectin can serve as a binding site for the $\alpha_v\beta_5$ integrin and that the integrin binding requires an appropriate sequence of basic amino acids. These results suggest the existence of a previously unrecognized integrin specificity directed toward a sequence motif consisting of several basic amino acids.

Materials and Methods

Peptide Synthesis

The intact Tat protein was synthesized using t-butoxycarbonyl (BOC)-protected amino acids for stepwise synthesis on a solid phase automated peptide synthesizer (model 431A, Applied Biosystems, Inc., Foster City, CA). Amino acids were added as hydroxybenzotriazole (HOBt) esters using *n*-methylpyrrolidone as the coupling solvent. The synthesis was accomplished starting with 0.5 mmol of Boc-Glu(OBzl)-O-phenylacetamidomethyl resin (0.69 g substituted at 0.72 mmol) with a minimum of two couplings

for each amino acid. The average repetitive coupling efficiency was 99.32% as determined by a quantitative ninhydrin assay. The cysteine sulfhydryls were protected with a p-methylbenzyl group to yield a fully-reduced form after low/high HF cleavage using suitable scavengers. All other peptides used were synthesized with a synthesizer (model 430A, Applied Biosystems, Inc.) using similar chemistry. The Tat protein sequence:

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1          10          20          30          40
MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRK

           60          70          80
KRRQRRRAHQNSQTHQASLSKQPTSQSRGDPTGPKE,
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(Arya et al., 1985) was used in the peptide syntheses. In the Tat 45–86 peptide, a lysine residue was added to the NH_2 terminus to facilitate solid phase coupling. For the same reason, residue 57 in Tat 57–86 was changed from arginine to lysine, and an NH_2-terminal cysteine was added to the vitronectin basic domain peptide. In Tat 47–58, residue 58 was proline as in a variant Tat sequence (Green and Loewenstein, 1988).

Cell Adhesion

Cell attachment assays were performed essentially as described (Ruoslahti et al., 1982). Microtiter plates (96 well) were coated with substrate for 1 h in the presence of 0.25% glutaraldehyde. The plates were washed and then blocked with 1 M ethanolamine containing 2.5 mg/ml BSA. Subconfluent rat L8 or human SK-LMS cells were detached from their substrate with trypsin as described (Brake et al., 1990), washed three times with 0.5 mg/ml soybean trypsin inhibitor and resuspended in DME at 10^6 cells/ml. 100 μl of cell suspension was added to each well in the presence or absence of inhibitory peptides or antibodies. After a 1-h incubation, the attached cells were fixed in 3% paraformaldehyde and stained with 0.5% crystal violet. The dye was eluted from the stained cells with 100 μl of 50% ethanol containing 100 mM sodium citrate (pH 4.2). Attachment was quantified by reading the absorbance at 600 nM.

Affinity Chromatography

The Tat-binding proteins were isolated from surface-iodinated cells essentially as described (Pytela et al., 1985*a*,*b*). Cells were detached from culture plates in 100 μg/ml trypsin (Sigma Chemical Co., St. Louis, MO) as in the cell adhesion assays, and washed three times in 500 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.). Cells were surface iodinated using lactoperoxidase and extracted with a buffer containing 150 mM octyl glucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.4 μg/ml pepstatin, 150 mM NaCl and 50 mM Tris, pH 7.4. The extracts were clarified at 15,000 g and passed over a column containing Tat peptides coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) or peptides coupled to thiopropyl Sepharose (Pharmacia LKB Biotechnology, Inc.) through an amino-terminal cysteine. After an incubation of 2 h, the column was washed with several volumes of extraction buffer containing 50 mM octyl glucoside. The bound receptor was then eluted with the indicated peptide at a concentration of 1 mg/ml in the buffer used to wash the column unless otherwise noted. Aliquots were used for immunoprecipitation or were boiled in electrophoresis sample buffer and run on 7.5% SDS-polyacrylamide gels.

Antibodies

Polyclonal antibodies against the cytoplasmic tails of α_v, β_3, and β_5 were raised in rabbits by immunization with synthetic peptides coupled to keyhole limpet hemocyanin. The peptides used were KRVRPPQEEQEREQLQPHENGEGNSET from the COOH terminus of α_v (Suzuki et al., 1986), KFEEERARAKWDTANNPLYKEATSTFTNITYRGT from the COOH terminus of β_3 (Fitzgerald et al., 1987), and KKPISTHTVDFTFNKSYNGTVD from the COOH-terminus of β_5 (Suzuki et al., 1990). All peptides were synthesized with a synthesizer (model 430A, Applied Biosystems, Inc.). Additional details on the preparation of some of these antibodies have been published (Freed et al., 1989). The anti-β_1 subunit antiserum has also been described (Giancotti and Ruoslahti, 1990). Each of the antibodies was shown to bind to the appropriate integrin subunit in immunoblotting and to immunoprecipitate integrins containing these subunits from various surface-labeled cell lines.

Polyclonal antibodies prepared against the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins have been described (Argraves et al., 1987; Suzuki et al., 1986). mAbs were a gift of Dr. David Cheresh (The Scripps Research Institute, La Jolla, CA) (LM 609; Cheresh and Spiro, 1987) or Bristol Myers-Squibb Pharmaceutical Research Institute (Seattle, WA) (P3G2; Wayner et al., 1991).

Immunoprecipitations

Immunoprecipitations were performed by incubating material in the presence of 5 μl of immunized rabbit serum and 50 μl protein A Sepharose for 1 h. The receptor-antibody-protein A complex was spun down and washed three times with 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.4. The complex was then boiled in electrophoresis sample buffer and loaded on 7.5% SDS-polyacrylamide gels.

Results

Cell Adhesion to Tat

To investigate the mechanism of cell interaction with the Tat protein we performed cell attachment assays with a variety of Tat derived peptides. L8 rat skeletal muscle cells were chosen for these experiments because they had been shown to bind to Tat in a previous study (Brake et al., 1990). In agreement with the earlier study (Brake et al., 1990), L8 cells readily attached to the Tat protein and to a Tat peptide that contained the RGD sequence in addition to the basic domain (Tat 45–86). Also in agreement with the results of Brake et al. (1990) was that the cells did not spread on the surface. An unexpected result was that the cells did not attach to a shorter peptide in which the basic region was deleted (Tat 57–86 with residue 57 changed from arginine to lysine), even though this peptide contained the cell attachment sequence RGD (Fig. 1 *A*). Similar results were obtained with the human leiomyosarcoma cell line, SK-LMS (Fig. 1 *B*); the cells bound only to those peptides that contained the basic region. A peptide containing only the basic domain and three flanking amino acids (residues 47–58) supported cell attachment as well as full-length Tat on a molar basis even though this peptide did not contain the RGD sequence or the region flanking it. These results, together with the result that the attachment of cells to Tat was inhibited by heparin (see Table I), suggest that the basic region of Tat is required for cell adhesion, and that the RGD-containing region of Tat by itself is incapable of supporting adhesion of the cells tested.

Isolation and Identification of Tat-binding Proteins

To identify the integrins or other cell surface molecules capable of binding to Tat, affinity chromatography was performed using the 86-amino acid Tat protein and the Tat peptides. As was the case in the cell attachment experiments shown in Fig. 1, all of the peptides that contained the basic domain of Tat were active and gave similar results with the L8 and SK-LMS cells. Shown in Fig. 2 are the proteins from an iodinated L8 cell extract that bound to the Tat 45–86 peptide and eluted with this peptide or with full-length Tat. Bands of 150 kD and a doublet at ~90 kD were eluted from the column. Changing the RGD sequence to KGE (lys-gly-glu) or deleting the second exon entirely had no discernible effect on the identity of the proteins eluted from the column. Even the 12 amino acids comprising the basic domain were sufficient to bind and elute these bands (see Fig. 3). However, peptides lacking the basic domain did not bind significant amounts of iodinated cell surface proteins or elute proteins from columns that were active (not shown).

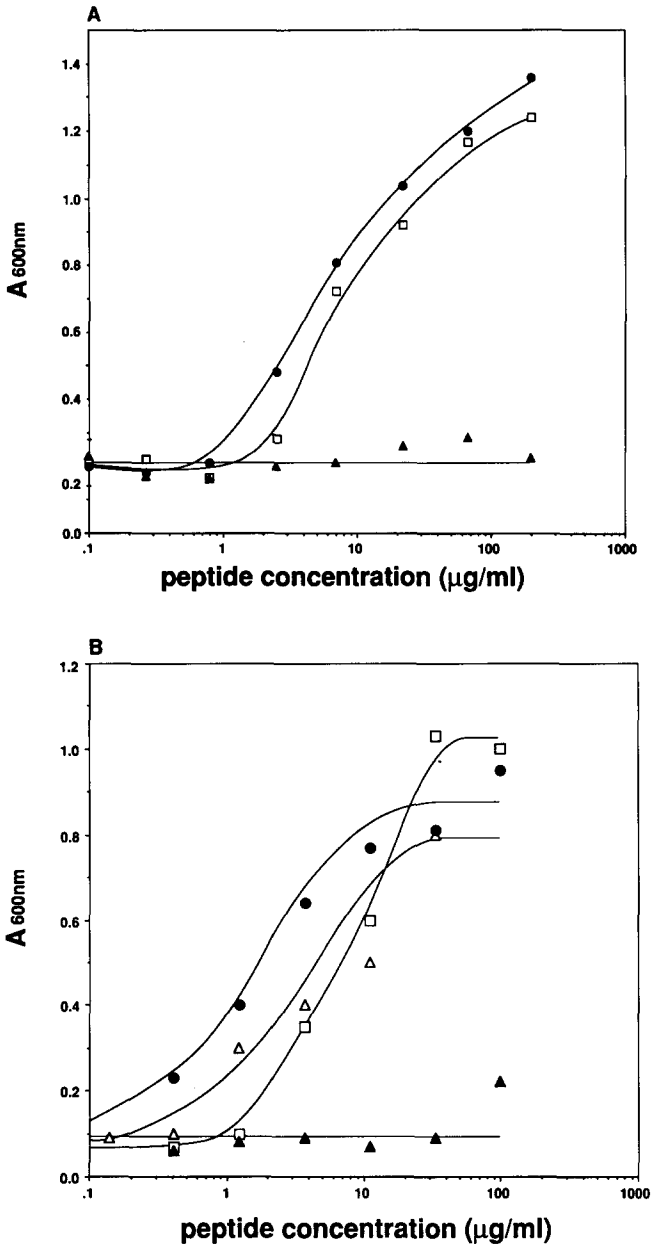

Figure 1. Cell adhesion to Tat peptides. Wells of microtiter dishes were coated with the indicated concentrations of Tat 1–86 (□), Tat 45–86 (●), Tat 57–86 (▲), or Tat 47–58 (△). After blocking nonspecific binding sites, ~10^5 cells were added to each well and incubated at 37°C for 1 h. The attached cells were fixed with paraformaldehyde and stained with crystal violet. The dye was eluted and the absorbance at 600 nm measured in an ELISA reader. L8 cells were tested on the first three peptides (*A*) and SK-LMS cells on each of the four peptides (*B*). Maximum cell attachment was ~8 × 10^4 cells/well. Each point represents the average of three independent experiments. The standard deviation for each point was 0.15 or less. The sequence of the Tat protein used in this work is from the HXB-2 strain (Arya et al., 1985):

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1          10         20         30         40
MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCF I TKALG I S

  50         60         70         80
YGRKKRRQRRRAHQNSQTHQASLSKQPTSQSRGDPTGPKE
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Table I. Adhesion of L8 Cells to Vitronectin and Tat

Adhesion of L8 cells to	In the presence of			
	1 mM GRGDSP	300 nM NaCl	0.5 mg/ml heparin	10 mM EDTA
Vitronectin	−	+	+	−
Tat	+	−	−	+

Adhesion assays were performed as described in Experimental Procedures. + indicates adhesion was significantly above background binding. − indicates background binding levels of adhesion.

Immunoprecipitation showed that the material bound to the Tat column consisted primarily of integrins. Fig. 3 shows an immunoprecipitation of surface-iodinated SK-LMS cell proteins eluted from the Tat 45-86 peptide column. The column was eluted sequentially with the peptide GRGDSP followed by a 12-amino acid peptide containing the basic domain of Tat. A small portion of the bound material eluted with the peptide GRGDSP and could be immunoprecipitated with β_1 and β_3 antibodies. However, the majority of the β_1 and β_3 subunit-associated material did not bind to the column and was detected in the unbound fraction. These results indicate that β_1 and β_3 integrins bind poorly to the column despite the presence of an RGD sequence in the pep-

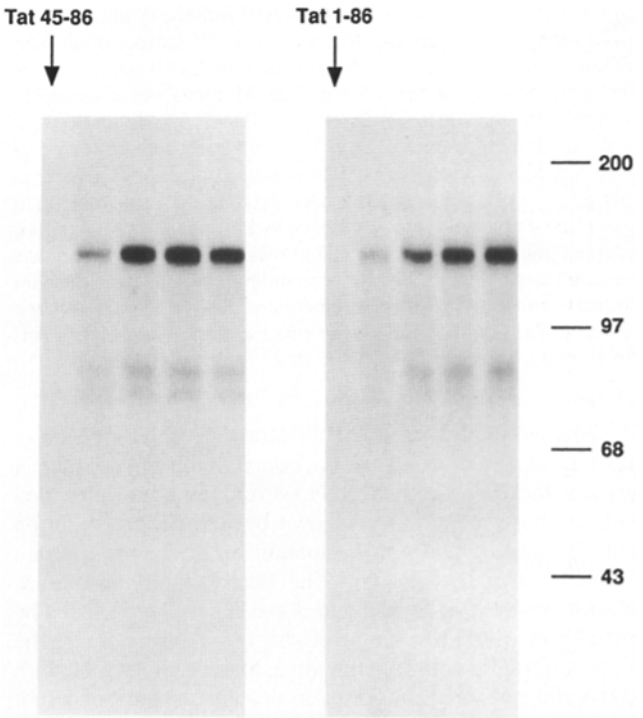

Figure 2. Affinity chromatography on Tat peptide. Approximately 5 × 10^7 rat L8 cells were detached from culture dishes with 0.1 mg/ml trypsin and washed three times with 0.5 mg/ml soybean trypsin inhibitor. The cells were iodinated using lactoperoxidase and extracted with octylglucoside (see Materials and Methods). The iodinated L8 cell extract was fractionated on Tat peptide 45–86 coupled to Sepharose. After washing, the column was eluted with either 1 mg/ml of the Tat peptide or 200 μg/ml of the full-length Tat protein. The fractions were analyzed by SDS PAGE (7.5%) under nonreducing conditions.

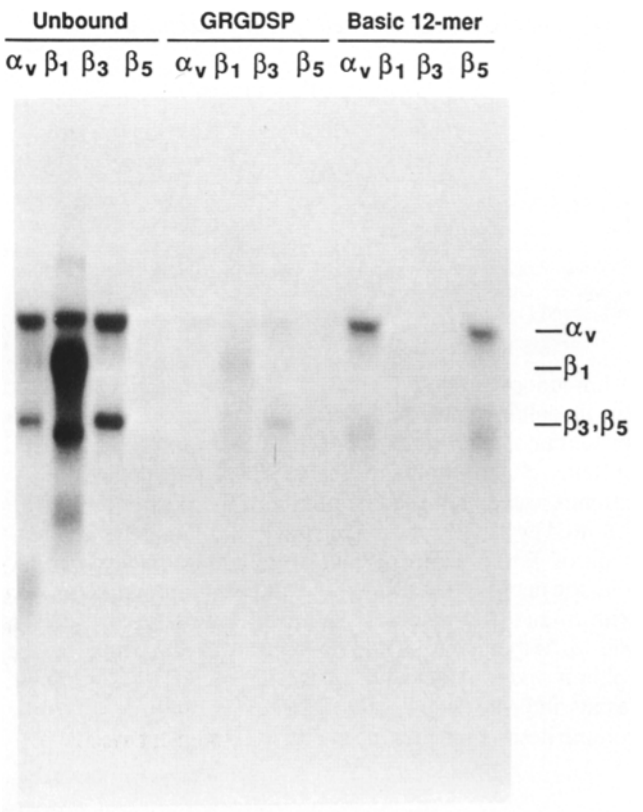

Figure 3. Immunoprecipitation of material isolated by affinity chromatography on Tat peptide. An octylglucoside extract of surface-iodinated SK-LMS cells was fractionated on Tat 45–86 coupled to Sepharose as described in Materials and Methods. After sequential elution with GRGDSP, Tat 57–86 and Tat 47–58 (basic 12-mer), peak fractions of each eluate were immunoprecipitated with 5 μl of serum containing polyclonal antibodies to the α_v, β_1, β_3, or β_5 subunit cytoplasmic domains and protein A Sepharose. The precipitated proteins were solubilized by boiling in SDS-containing sample buffer and analyzed by SDS PAGE (7.5%) under nonreducing conditions. Shown are the immunoprecipitations of the flow through (unbound), GRGDSP eluate and Tat 47–58 eluate (basic 12-mer). The antibody used is indicated at the top of each lane and the subunits are identified on the side.

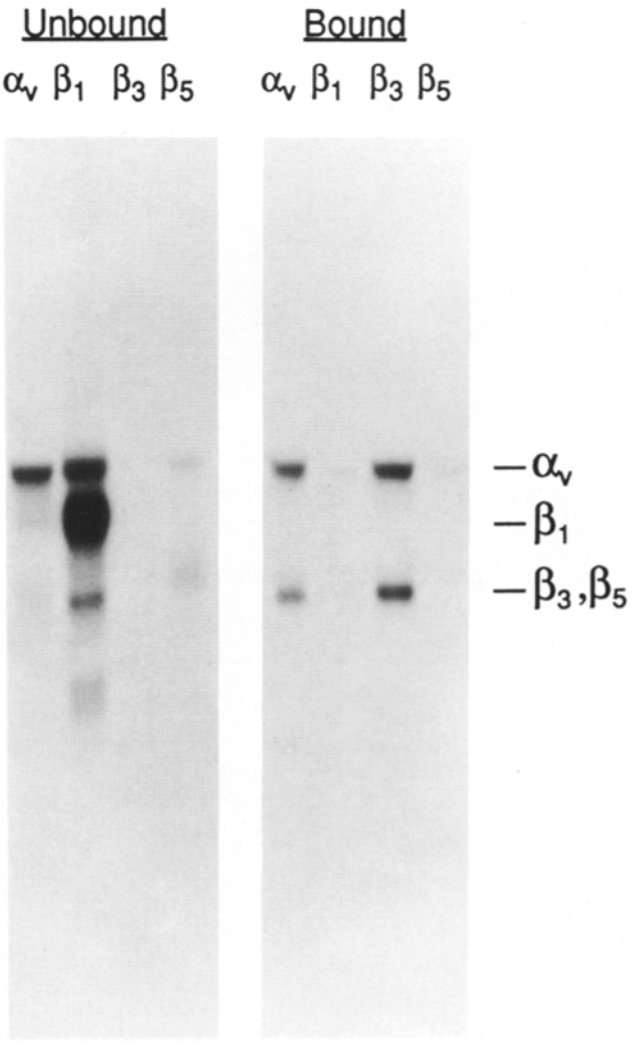

Figure 4. Immunoprecipitation of material affinity-purified on GRGDSPK Sepharose. An extract of surface-iodinated SK-LMS cells was fractionated on GRGDSPK Sepharose as described in Materials and Methods. After elution with GRGDSP (1 mg/ml), the flow through (unbound) and eluate (bound) was immunoprecipitated with antibodies to the α_v, β_1, β_3, or β_5 subunit cytoplasmic domains (see Fig. 3, *legend*), and analyzed by SDS PAGE (7.5%) under nonreducing conditions.

tide coupled to the column. They also suggest that the material that remained bound was not bound to the column matrix through the RGD sequence. However, the material eluted with the basic peptide was shown by immunoprecipitation with the anti-α_v and anti-β_5 subunit antibodies to contain the $\alpha_v\beta_5$ integrin (Fig. 3). The heterogeneity of the β_5 subunit apparent in Figs. 2 and 3 was most likely caused by partial proteolysis resulting from harvesting the cells for the chromatography with trypsin, since material harvested with EDTA did not yield the lower molecular weight band (not shown). These results, together with the cell attachment assays, indicate that our test cells bind to the basic domain of the Tat protein and that this binding is mediated by the $\alpha_v\beta_5$ integrin.

Comparison of Integrins Bound to Tat with Those Bound to GRGDSPK Peptide

Affinity chromatography of the SK-LMS cell extracts on the peptide GRGDSPK coupled to Sepharose (Fig. 4) revealed a strikingly different pattern than the one obtained with the Tat peptide. Whereas the predominant integrin binding to the Tat Sepharose was $\alpha_v\beta_5$, the $\alpha_v\beta_3$ integrin was the major integrin enriched in the GRGDSPK-bound fraction (Fig. 4). Although some $\alpha_v\beta_5$ was observed in the bound fraction, the majority of this integrin was in the unbound fraction. This is in agreement with earlier results showing that $\alpha_v\beta_5$ has a relatively weak affinity for the GRGDSPK peptide (Freed et al., 1989). Together, these data show that although SK-LMS cells contain functional $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins capable of binding the GRGDSPK peptide, the RGD sequence in Tat is present in a context unfavorable to the binding of these integrins.

Integrin Binding to Peptides Consisting Entirely of Basic Amino Acids

The Tat basic domain contains a single glutamine flanked by

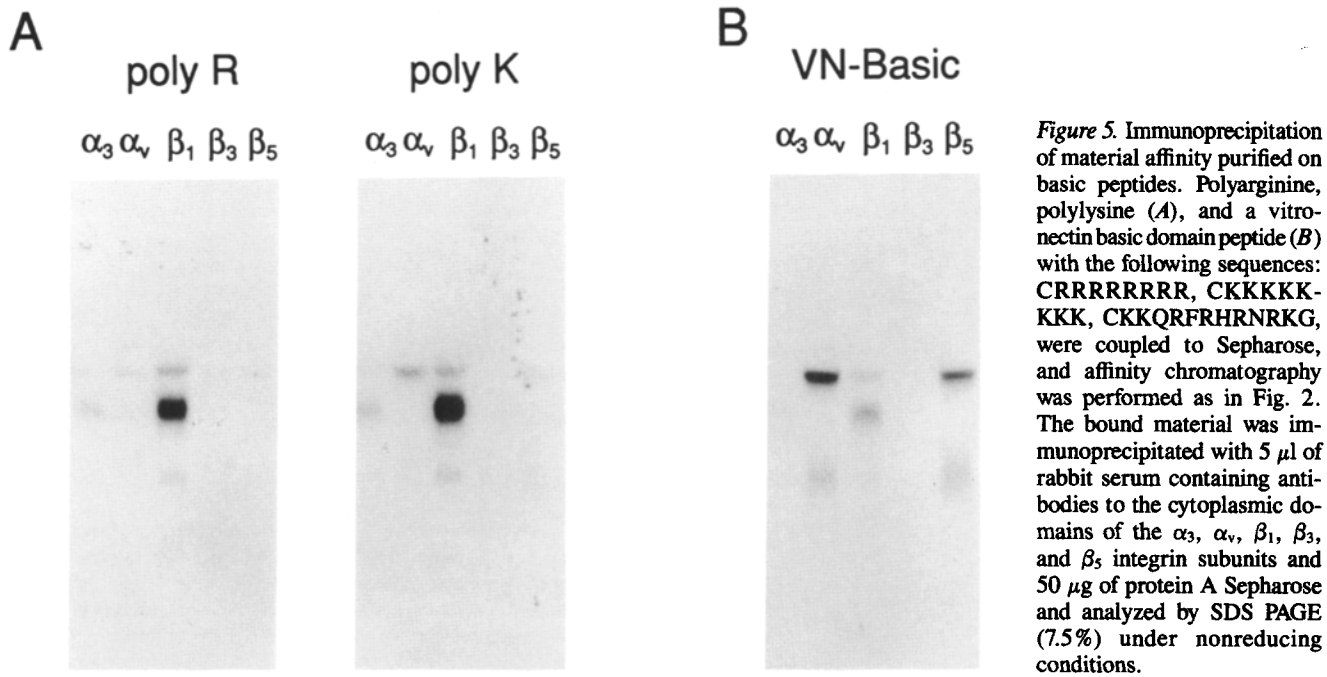

Figure 5. Immunoprecipitation of material affinity purified on basic peptides. Polyarginine, polylysine (*A*), and a vitronectin basic domain peptide (*B*) with the following sequences: CRRRRRRRR, CKKKKKKKK, CKKQRFRHRNRKG, were coupled to Sepharose, and affinity chromatography was performed as in Fig. 2. The bound material was immunoprecipitated with 5 μl of rabbit serum containing antibodies to the cytoplasmic domains of the α_3, α_v, β_1, β_3, and β_5 integrin subunits and 50 μg of protein A Sepharose and analyzed by SDS PAGE (7.5%) under nonreducing conditions.

two arginine residues on the NH_2-terminal side and three on the COOH-terminal side. To determine if the $\alpha_v\beta_5$ interaction was specific for the Tat basic domain or if $\alpha_v\beta_5$ would bind to any arginine-rich peptide, affinity chromatography was also performed with a peptide, CRRRRRRRR, consisting entirely of arginine residues (with an NH_2-terminal cysteine added for coupling). A similar peptide consisting entirely of lysine residues was also used (CKKKKKKKK). The $\alpha_v\beta_5$ integrin was not detectable in the bound fraction from columns containing either of these peptides (Fig. 5 *A*). Instead, the β_1 subunit was the prominent immunoprecipitable integrin subunit from the bound fractions of both columns. Analysis of the β_1 integrins bound to these columns revealed that α_6 and α_5 were the major subunits bound to the arginine-rich columns and lysine-rich columns, respectively (not shown), whereas α_3 bound to both columns (Fig. 5 *A*).

Binding of $\alpha_v\beta_5$ to a Basic Domain Sequence from Vitronectin

To test whether the interaction of the basic domain of Tat with $\alpha_v\beta_5$ was representative of an interaction between $\alpha_v\beta_5$ and its ligand, vitronectin, affinity chromatography was performed with a peptide representing a portion of the vitronectin heparin-binding domain (CKKQRFRHRNRKG). The results were similar to those with the Tat peptides, except that less integrin appeared to bind to the vitronectin peptide than to Tat. The majority of the bound material was precipitable with α_v and β_5 antibodies, although some anti-β_1-reactive material was also detected (Fig. 5 *B*). This suggested that $\alpha_v\beta_5$ can bind to the heparin-binding domain of vitronectin in addition to its previously characterized interaction with the RGD sequence of vitronectin (Freed et al., 1989; Cheresh et al., 1989). Next, the basic peptides from vitronectin and from Tat were used to inhibit cell attachment to vitronectin. As shown previously (Cheresh et al., 1989), the GRGDSP peptide was capable of weakly inhibiting the $\alpha_v\beta_5$-mediated attachment of cells to vitronectin (Fig. 6). Although the basic peptides by themselves were not effective inhibitors of L8 cell adhesion to vitronectin, they enhanced the inhibitory activity of GRGDSP when added together with it (Fig. 6).

Characteristics of $\alpha_v\beta_5$ Basic Domain Interactions

To characterize what appeared to be an atypical integrin-ligand interaction, we studied the sensitivity of the $\alpha_v\beta_5$ basic sequence interaction to high salt, certain antibodies and

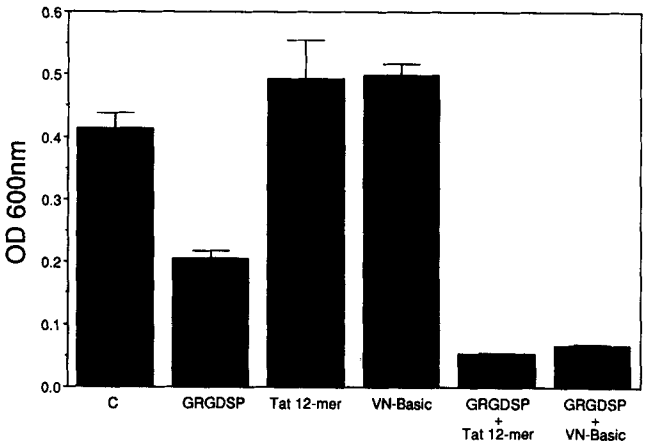

Figure 6. Inhibition of cell attachment to vitronectin with peptides. L8 cells were allowed to attach to microtiter wells coated with 10 μg/ml vitronectin in the presence or absence of 0.5 mM GRGDSP Tat basic domain peptide, vitronectin (*VN*) basic domain peptide, or combinations of GRGDSP with one of the basic peptides. Attached cells were fixed and stained as described in Materials and Methods. Maximum cell adhesion was ~80% of the 10^5 cells added to each well. Each value represents the average of three independent experiments. Error bars indicate the standard deviation for each point.

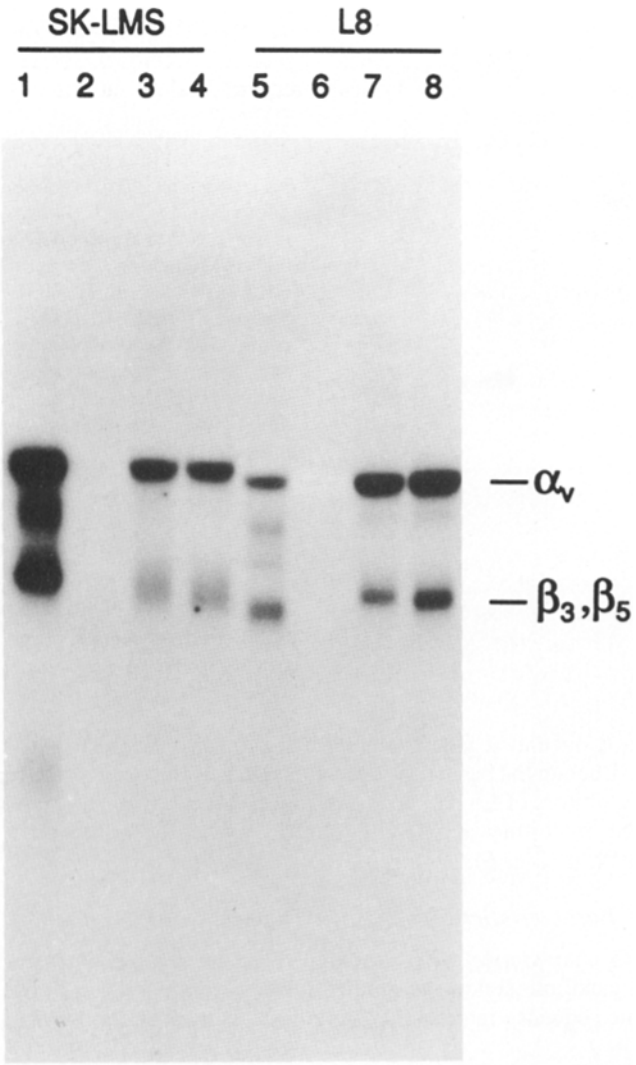

Figure 7. Elution of integrin from Tat column with EDTA or NaCl. Extracts of surface-iodinated L8 or SK-LMS cells were fractionated on Tat 45–86 coupled to Sepharose. Sequential elution of the columns with 10 mM EDTA (in 100 mM NaCl; lanes *2* and *6*), 250 mM NaCl (lanes *3* and *7*) and Tat 45–86 (1 mg/ml; lanes *4* and *8*), followed by immunoprecipitation with a polyclonal anti-$\alpha_v\beta_3$ antibody and analysis by SDS PAGE (7.5%) under nonreducing conditions are shown. Lanes *1* and *5* are immunoprecipitates of the flow-through material.

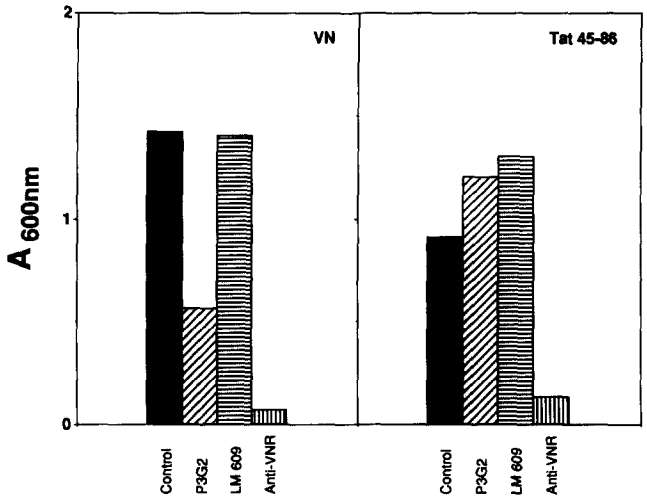

Figure 8. Inhibition of SK-LMS cell attachment to vitronectin and Tat peptide. Approximately 10^5 SK-LMS cells were added to microtiter wells previously coated with 10 μg/ml vitronectin (*VN*) or Tat 45–86 in the presence or absence of inhibitory antibodies. P3G2 is a mAb shown previously to inhibit the interaction of $\alpha_v\beta_5$ with vitronectin (Wayner et al., 1991). LM 609 inhibits the interaction of $\alpha_v\beta_3$ with vitronectin (Cheresh and Spiro, 1987). Anti-VNR is a polyclonal antibody raised against the $\alpha_v\beta_3$ integrin purified from a placental extract on a GRGDSPK-Sepharose column (Pytela et al., 1987; Freed et al., 1989). P3G2 was culture supernatant diluted 1:2. LM 609 was a partially purified IgG fraction used at 3 μg/ml. The anti-VNR is a rabbit serum diluted 1:20. All of these concentrations were tested for function previously and were not toxic to the cells. After a 1-h incubation at 37°C, the attached cells were fixed and stained as described above. Each point represents the mean of three independent experiments with a standard deviation smaller than 0.2 absorbance units.

removal of divalent cations. We used the Tat 45–86 peptide for these studies, because it bound $\alpha_v\beta_5$ more effectively than the basic peptide from vitronectin. Integrins typically require divalent cations to bind their ligands and can be eluted from ligand affinity columns with EDTA (Pytela et al., 1987). However, the interaction between $\alpha_v\beta_5$ and Tat was insensitive to elution with 10 mM EDTA in affinity chromatography experiments (Fig. 7). Immunoprecipitations of the peak fractions eluted with 10 mM EDTA, 250 mM NaCl, or the Tat 45–86 peptide with a polyclonal anti-$\alpha_v\beta_3$ antibody revealed no $\alpha_v\beta_5$ in the EDTA-eluted fractions, whereas some of it eluted in the high salt fractions and the rest was released by the Tat peptide elution. We have also performed cell adhesion experiments in the presence of various inhibitors; the results are summarized in Table I.

Because the binding of the basic domain peptides to $\alpha_v\beta_5$ was a novel interaction, we wanted to determine if the basic peptides bound to the same site on $\alpha_v\beta_5$ that vitronectin, a principal $\alpha_v\beta_5$ ligand, does. The mAb P3G2 had previously been shown to bind to $\alpha_v\beta_5$ and to inhibit its interaction with vitronectin (Wayner et al., 1991). We were able to reproduce this result using SK-LMS cells as shown in Fig. 8. However, the mAb did not inhibit the binding of the cells to Tat. Our polyclonal anti-$\alpha_v\beta_3$ antibody did inhibit the attachment of the SK-LMS cells (Fig. 8) and L8 cells (not shown) to both Tat and vitronectin, indicating that a receptor related to $\alpha_v\beta_3$ mediates the interaction between SK-LMS cells and Tat. A function-inhibiting mAb recognizing $\alpha_v\beta_3$ (LM 609; Cheresh and Spiro, 1987) did not inhibit the binding of the cells to either Tat or vitronectin. Taken together with the affinity chromatography results, these findings indicate that the attachment of the SK-LMS cells to vitronectin is mediated by $\alpha_v\beta_5$ and that two distinct regions in the integrin are involved, one binding to the RGD region and the other to the basic domain.

Discussion

The findings reported here identify a novel interaction between integrins and the basic domains of certain adhesive proteins. We were led to this observation while working on the previously noted ability of certain cells to attach to the HIV Tat protein (Brake et al., 1990). Tat can function as an

exogenous factor to alter cellular gene expression and modulate cell proliferation (Viscidi et al., 1989; Ensoli et al., 1990), and the basic region of Tat has been found to be important for the binding of Tat to the cell surface (Mann and Frankel, 1991). As Tat had previously been claimed to exert a cell attachment-promoting activity through its RGD sequence (Brake et al., 1990), integrins were obvious candidates for mediation of this activity. We found that the $\alpha_v\beta_5$ integrin mediates the cell attachment activity of Tat. However, anti-integrin antibodies capable of blocking cell attachment to Tat were not able to block uptake of Tat into L8 cells (our unpublished results), making it unlikely that the $\alpha_v\beta_5$ integrin would play a role in Tat internalization by cells. Rather, the interaction of Tat with $\alpha_v\beta_5$ appears to be representative of a more general and potentially physiological, activity of the integrins.

The assignment of $\alpha_v\beta_5$ as the Tat-binding integrin was based on immunological identification: The integrin isolated by chromatography on Tat columns from the human SK-LMS cells was reactive with antibodies prepared against the cytoplasmic domains of the α_v and β_5 subunits, and polyclonal antibodies that bind to the α_v subunit in $\alpha_v\beta_5$ inhibited the attachment of these cells to the Tat-coated surface. This result agrees with the assumption that the $\alpha_v\beta_5$ integrin is the receptor that mediates the attachment of the SK-LMS cells to Tat.

Although the Tat-binding integrin on the human SK-LMS cells was identified as $\alpha_v\beta_5$, we were unable to positively identify the β subunit of the Tat binding integrin from the rat L8 cells. It seems likely that this integrin is also $\alpha_v\beta_5$ because it behaved identically to the human $\alpha_v\beta_5$ integrin in the affinity chromatography experiments, and its β subunit migrated similarly to the human β_5 in SDS-PAGE. However, the β subunit reacted, at best, weakly with our anti-β_5 antibodies. These antibodies were prepared against the cytoplasmic peptide of the human β_5 subunit and were poorly reactive when tested against a number of rat cell lines. Poor reactivity of the antibodies with the rat β_5 subunit may therefore explain the lack of immunoprecipitation of the L8 integrin. It is also possible that the β subunit of the Tat binding integrin from the L8 cells may be an alternatively spliced β_5 variant or a different β subunit altogether.

Although Tat contains an RGD sequence, and the $\alpha_v\beta_5$ integrin has been shown to recognize the RGD sequence in vitronectin and in some peptides (Cheresh et al., 1989; Freed et al., 1989; Smith et al., 1990), our results show that the integrin recognition sequence in Tat is the basic domain of Tat, not RGD. This conclusion is based on the complete correlation we found between the presence of the basic domain in the various Tat peptides and their ability to support cell attachment and to bind the $\alpha_v\beta_5$ integrin in affinity chromatography. In contrast, the presence or absence of the RGD sequence had no influence in either type of assay. Apparently, the RGD sequence is present in a context not suitable for binding any of the integrins present on the cells we used, because even the $\alpha_v\beta_3$ integrin, which is the best binder of short RGD-containing peptides (e.g., Fig. 4), failed to bind appreciably to the RGD-containing Tat peptides. The binding of the Tat basic domain by the $\alpha_v\beta_5$ integrin, therefore, appears to be a function distinct from the RGD binding.

In addition to utilizing a basic domain, the new interaction of $\alpha_v\beta_5$ has other unusual features. First, it is stable in the presence of 10 mM EDTA, whereas other integrin-ligand interactions, including the binding of $\alpha_v\beta_5$ to the RGD sequence of vitronectin, typically require divalent cations and are inhibited by the presence of EDTA (Pytela et al., 1987; Busk et al., 1992). Another characteristic of the basic domain binding of the $\alpha_v\beta_5$ integrin is that it was inhibited by NaCl concentrations above the physiological concentration. Although the concept of a salt-sensitive binding site for a basic peptide is unusual for integrin-ligand interactions, it is not without precedent. The $\alpha_3\beta_1$ integrin binds to collagen, fibronectin and laminin; the collagen and fibronectin binding is salt-sensitive (Wayner and Carter, 1987) and may therefore be equivalent to the binding of $\alpha_v\beta_5$ to basic sequences demonstrated here. In fact, the binding of $\alpha_3\beta_1$ to laminin has been found to be mediated by a basic sequence in laminin (Gehlsen et al., 1992). Furthermore, a recent study suggests that integrin-mediated binding of avian neural crest cells to laminin can be independent of divalent cations (Lallier and Bronner-Fraser, 1991), which is another characteristic shared by the basic sequence binding site in $\alpha_v\beta_5$.

These differences between the binding sites for the RGD and basic sequences in $\alpha_v\beta_5$, in addition to the fact that we were unable to inhibit basic domain-mediated cell attachment with a mAb that inhibits the RGD-mediated binding of $\alpha_v\beta_5$ to vitronectin, suggest that this integrin has two separate binding sites. The concept of two distinct ligand binding sites has been suggested for the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins. Each of these two integrins has a binding site for the endothelial cell ligand, V-CAM, and another binding site for an alternatively spliced segment of fibronectin (Elices et al., 1990; Rüegg et al., 1992). The data on the $\alpha_3\beta_1$ integrin discussed above and our finding of β_1 integrin binding to peptide columns comprised entirely of arginine or lysine also support the notion that at least some of the β_1 integrins also contain a site for basic domain binding. Among the β_1 integrins that bound to the polyarginine and/or polylysine columns were $\alpha_5\beta_1$ and $\alpha_3\beta_1$; $\alpha_5\beta_1$ binds also to RGD (Pytela et al., 1985*a*) and the same has been reported for $\alpha_3\beta_1$ (Elices et al., 1991). Finally, IIb/IIIa may also share some of the RGD and basic peptide binding properties of $\alpha_v\beta_5$, because peptides containing both an RGD and a basic segment bind more avidly to IIb/IIIa than peptides containing RGD alone (Savage et al., 1990). Therefore, all integrins may have an RGD (or equivalent) binding site in addition to a basic sequence (or equivalent) binding site.

The physiological significance of the basic sequence binding by the $\alpha_v\beta_5$ integrin remains to be elucidated, but two findings among our results indicate such a role. First, this binding appeared to make a contribution to cell attachment in vitro, because the attachment of our test cells to vitronectin was inhibited to a substantially greater degree by a combination of a basic peptide and an RGD peptide than by the RGD peptide alone. Secondly, the basic domain binding displayed a specificity; the $\alpha_v\beta_5$ integrin was the only integrin that bound substantially to the Tat basic domain peptide, whereas peptides consisting of only arginine or lysine residues as the basic amino acid primarily bound β_1 integrins. This suggests that the only nonbasic residue, a single glutamine, in the basic nine amino acid stretch of Tat may be important for the specificity of Tat toward $\alpha_v\beta_5$. This assumption is supported by the fact that the peptide from the basic domain of vitronectin also bound $\alpha_v\beta_5$, because the

vitronectin peptide also contains a glutamine residue and, as is the case with the Tat peptide, this residue is surrounded by basic residues (KKQR in vitronectin vs. RRQR in Tat). Because the arrangement of the basic amino acids in the two peptides is otherwise quite different, the exact order of the basic amino acids may not be important. The specificities of the basic domain interactions, therefore, do not seem to be as clear-cut as with some other receptor-ligand interactions.

Whereas the Tat peptide displayed specificity for $\alpha_v\beta_5$, especially in the affinity chromatography experiments, that of the vitronectin peptide was intermediate between the Tat peptide and the peptides consisting entirely of arginine or lysine residues. We also found even cells lacking the $\alpha_v\beta_5$ integrin to be capable of attaching to Tat (not shown). This situation is reminiscent of the RGD system where various RGD-directed integrins also display much overlap in specificity (Ruoslahti, 1991).

Finally, it may be that the vitronectin peptide is not fully representative of the binding site in vitronectin, either because it does not represent the entire site or because the conformation of the site as a peptide is not the same as in the intact protein. An intriguing possibility is that the Tat-derived basic peptide is more active than the peptide from the vitronectin basic domain, because it happens to mimic a basic domain in an $\alpha_v\beta_5$ ligand yet to be found.

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