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-aspartate (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 $\alpha_v$ 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 $\alpha_\beta_1$ 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-aspartate (RGD) 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, $\alpha$ and $\beta$ (Hemler, 1990; Ruoslahti, 1991; Hynes, 1992).

The Tat protein of human immunodeficiency virus (HIV-I) 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).

We felt that the possibility of an RGD-binding integrin mediating some of the interactions of Tat with cell surfaces was of considerable interest and set out to identify such an integrin. We found that the $\alpha_v\beta_5$ integrin bound to Tat, 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 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 hydroxymethylribazole (HOBt) esters using n-methylpyrrolidine 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 sulfoxyls 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: GQQQQQQKRRQRRRAHQNSQTHQASLSKQPTSQSRGDPTG PKE,

(KRVRPPQEEQEREQLQPH-arginine to lysine, and an NH2-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 (Russilati 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 by trypsin as described (Brake et al., 1990), washed three times with 0.5 mg/ml soybean trypsin inhibitor and resuspended in DMEM at 106 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., 1985a,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 CaCl2, 1 mM MgCl2, 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 thiopropyl Sepharose 413 (Pharmacia LKB Biotechnology, Inc.) through an amino-terminal cysteine. 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 domain. 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 1), 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).
Table I. Adhesion of L8 Cells to Vitronectin and Tat

|               | 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.

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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 αv, β1, β3, or β5 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.

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 αvβ3, the αvβ3 integrin was the major integrin enriched in the GRGDSPK-bound fraction (Fig. 4). Although some αvβ3 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 αvβ3 has a relatively weak affinity for the GRGDSPK peptide (Freed et al., 1989). Together, these data show that although SK-LMS cells contain functional αvβ3 and αvβ5 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
two arginine residues on the NH2-terminal side and three on the COOH-terminal side. To determine if the αβ5 interaction was specific for the Tat basic domain or if αβ3 would bind to any arginine-rich peptide, affinity chromatography was also performed with a peptide, CRRRRRRRR, consisting entirely of arginine residues (with an NH2-terminal cysteine added for coupling). A similar peptide consisting entirely of lysine residues was also used (CKKKKKKK). The αβ5 integrin was not detectable in the bound fraction from columns containing either of these peptides (Fig. 5 A). Instead, the β3 subunit was the prominent immunoprecipitable integrin subunit from the bound fractions of both columns. Analysis of the β3 integrins bound to these columns revealed that αb3 and α5 were the major subunits bound to the arginine-rich columns and lysine-rich columns, respectively (not shown), whereas α1 bound to both columns (Fig. 5 A).

**Binding of αβ3 to a Basic Domain Sequence from Vitronectin**

To test whether the interaction of the basic domain of Tat with αβ3 was representative of an interaction between αβ3 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 αb3 and β3 antibodies, although some anti-β3-reactive material was also detected (Fig. 5 B). This suggested that αβ3 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 αβ3-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 αβ3 Basic Domain Interactions**

To characterize what appeared to be an atypical integrin-ligand interaction, we studied the sensitivity of the αβ3 basic sequence interaction to high salt, certain antibodies and...
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 αvβ3 with vitronectin (Wayner et al., 1991). LM 609 inhibits the interaction of αvβ5 with vitronectin (Cheresh and Spiro, 1987). Anti-VNR is a polyclonal antibody raised against the αvβ5 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.

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-αvβ3 antibody and analysis by SDS PAGE (7.5%) under nonreducing conditions are shown. Lanes 1 and 5 are immunoprecipitates of the flow-through material.

removal of divalent cations. We used the Tat 45–86 peptide for these studies, because it bound αvβ3 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 αvβ3 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-αvβ3 antibody revealed no αvβ3 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 αvβ3 was a novel interaction, we wanted to determine if the basic peptides bound to the same site on αvβ3 that vitronectin, a principal αvβ3 ligand, does. The mAb P3G2 had previously been shown to bind to αvβ3 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-αvβ3 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 αvβ3 mediates the interaction between SK-LMS cells and Tat. A function-inhibiting mAb recognizing αvβ3 (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 αvβ3 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; Ensolli 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 αβ1 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 αβ1 integrin would play a role in Tat internalization by cells. Rather, the interaction of Tat with αβ2 appears to be representative of a more general and potentially physiological, activity of the integrins.

The assignment of αβ2, 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 and β subunits, and polyclonal antibodies that bind to the α subunit in αβ3 inhibited the attachment of these cells to the Tat-coated surface. This result agrees with the assumption that the αβ3 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 αβ3, 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 αβ3, because it behaved identically to the human αβ3 integrin in the affinity chromatography experiments, and its β subunit migrated similarly to the human β3 in SDS-PAGE. However, the β subunit reacted, at best, weakly with our anti-β antibodies. These antibodies were prepared against the cytoplasmic peptide of the human β3 subunit and were poorly reactive when tested against a number of rat cell lines. Poor reactivity of the antibodies with the rat β3 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 variant or a different β subunit altogether.

Although Tat contains an RGD sequence, and the αβ3 integrin has been shown to recognize the RGD sequence in vitronectin and in some peptides (Chereshe, 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 αβ3 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 αβ3 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 αβ3 integrin, therefore, appears to be a function distinct from the RGD binding.

In addition to utilizing a basic domain, the new interaction of αβ3 has other unusual features. First, it is stable in the presence of 10 mM EDTA, whereas other integrin-ligand interactions, including the binding of αβ3 to the RGD sequence of vitronectin, typically require divalent cations and are inhibited by the presence of EDTA (Pytel et al., 1987; Busk et al., 1992). Another characteristic of the basic domain binding of the αβ3 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 αβ3 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 αβ3 to basic sequences demonstrated here. In fact, the binding of αβ3 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 αβ3.

These differences between the binding sites for the RGD and basic sequences in αβ3, 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 αβ3 to vitronectin, suggest that this integrin has two separate binding sites. The concept of two distinct ligand binding sites has been suggested for the αβ6 and αβ2 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; Riegg et al., 1992). The data on the αβ3 integrin discussed above and our finding of β1 integrin binding to peptide columns comprised entirely of arginine or lysine also support the notion that at least some of the β1 integrins also contain a site for basic domain binding. Among the β1 integrins that bind to the polycarboxylate or polyllysine columns were αβ1 and αβ1; αβ1 binds also to RGD (Pytel et al., 1985a) and the same has been reported for αβ1 (Elices et al., 1991). Finally, IIb/IIIa may also share some of the RGD and basic peptide binding properties of αβ3, 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 αβ3 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 αβ3 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 β1 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 αβ3. This assumption is supported by the fact that the peptide from the basic domain of vitronectin also bound αβ3, 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 $\alpha_\beta$, 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 $\alpha_\beta$ 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 $\alpha_\beta$ ligand yet to be found.

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