A simple method for measurement of cell-substrate attachment forces: application to HIV-1 Tat

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SUMMARY

In order to understand the importance of cell attachment to HIV-1 Tat, we quantified the strength of cell attachment to immobilized Tat in microtiter plate wells by the application of buoyant force. By replacing the attachment medium with dense medium, and subjecting the attached cells in the microtiter plates to centrifugal force in the conventional upright position, weakly binding and strongly binding cells could be discriminated (and separated) by varying the centrifugal speed. The strength of attachment of HT1080 cells to Tat was compared with that of the well-known extracellular matrix (ECM) proteins fibronectin and vitronectin. We observed that all three proteins mediated significant attachment of HT1080 cells both at 4°C and 37°C. However, unlike the ECM proteins, Tat was unable to engage in higher strength binding when the temperature was raised to 37°C. The relatively weak binding of HT1080 cells to Tat (in the order of 3.0 dynes/picomole of coated Tat) and lack of strengthening of binding to Tat at physiological temperature suggests that this protein does not mimic adhesion molecule function. We anticipate that the methodology developed and described here will be useful in a wide variety of cell-matrix and cell-cell interaction studies.

Key words: Cell adhesion, Buoyant force, HIV-1 Tat

INTRODUCTION

Cell-cell and cell-matrix interactions play an essential role in physiological and pathological conditions such as cell motility, tissue differentiation, wound healing, inflammation and metastasis (reviewed by Diamond et al., 1994). These interactions occur as a result of specific recognition between adhesion molecules present on different cell types or between cells and extracellular molecules and can be qualitatively and quantitatively demonstrated in vitro. Adhesion of cells to a number of extracellular matrix proteins such as fibronectin (FN), vitronectin (VN), laminin, collagen etc., has been shown to induce cell spreading and formation of focal contacts, close contacts and stress fibers (Izzard and Lochner, 1976; Hynes and Destree, 1978; Singer, 1982; Virtanen et al., 1982; Woods et al., 1983; Burridge et al., 1988) indicating the strength of cell-substrate interactions. Some cell adhesion processes are known to involve an initial contact between cell and substrate, followed by an energy dependent strengthening of cell attachment (Juliano and Gagalang, 1977; Carter et al., 1981; Schwarz and Juliano, 1984; Loiz et al., 1989). Initial interactions which require specific recognition between receptor and ligand have been shown to be reversible (Umberg and Roseman, 1975). Such reversible interactions can be weaker than the shear forces generated by routine washing procedures generated in cell attachment assays which require careful separation of unattached cells from substrate bound cells.

Human immunodeficiency virus type-1 (HIV-1) Tat is an 86 amino acid transactivating protein and is encoded in two exons (Arya et al., 1985; Sodroski et al., 1985). The tat exon II product contains an RGD sequence at position 78-80. This sequence is also commonly found in the cell attachment domain of several extracellular matrix (ECM) molecules (reviewed by Humphries et al., 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Extracellular Tat has been shown to enter cells, localize in the nucleus and transactivate the HIV-1 long terminal repeat, suggesting the existence of a possible Tat receptor (Frankel and Pabo, 1988). Recent reports by Brake et al. (1990), Weeks et al. (1993), Vogel et al. (1993) and Barillari et al. (1993) and our own unpublished results have found that Tat promotes attachment of lymphocytes, skeletal muscle cells, endothelial cells and several other cell types, without causing cell spreading. However, the relevance of Tat mediated cell attachment to HIV-1 pathogenesis has not been well established. We were interested in characterizing cell-Tat interactions in terms of functional adhesion in order to understand the importance of cell attachment to Tat. We have studied the attachment of fibrosarcoma cells to Tat, and have compared it to that mediated by FN and VN. In order to do so we have employed an assay in which the unattached cells were removed by underlaying substrate bound cells with a medium of higher density than the cells, resulting in a buoyancy force on each cell that depends on the density difference, the cell volume, and the gravitational force. The strength of a cell-substrate attachment was determined by determining its resistance to increasing centrifugal force. Cells were labeled with a viability-
dependent fluorescent dye to ensure that only metabolically active cells were scored. In this paper, we show that while cell attachment to Tat did take place at 4°C and 37°C, the binding energies and temperature dependence were significantly different from FN and VN.

**MATERIALS AND METHODS**

**Cells**

HT1080 cells (human fibrosarcoma, CCL 121) purchased from ATCC, Rockville, MD were grown with MEM with Earle’s salts, non-essential amino acids, (from ABI, Advanced Biotecnologies, Columbia, MD), and 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin from Gibco BRL, Gaithersburg, MD.

**Peptides**

Human plasma FN (440 kDa) and human VN (75 kDa) were purchased from Life Technologies, Gibco. The 86 amino acid long HIV-1 Tat (10 kDa) protein was chemically synthesized by the solid-phase peptide synthesis method using a Peptide Synthesizer model #433A, Applied Biosystems Inc. (ABI, Foster City, CA). FMOC-protected amino acids were activated and coupled using the ABI FASTMOC procedure and 4-hydroxymethyl-phenoxyethyl resin. The basic procedure was modified to include trebling of the coupling time and double-coupling on all arginine residues. Side chain blocking groups were removed using the cleavage mixture B, consisting of trifluoroacetic acid (10 ml), water (0.5 ml), phenol (0.75 g), thioanisol (0.5 ml), and ethanedithiol (0.25 ml), as recommended by the manufacturer (Applied Biosystems). Tat protein was lyophilized and purified by reverse phase HPLC to >90% homogeneity. Authenticities of HPLC products were verified by mass spectrometry, amino acid analysis, and by electrophoresis in SDS-urea PAGE. Stock solutions of Tat, FN, and VN at 1 mg/ml were prepared in PBS and stored as aliquots at −20°C. Further dilutions of Tat, FN and VN were made in PBS.

**Adsorption isotherm of HIV-1 Tat onto polystyrene**

Synthetic HIV-1 Tat protein was labeled with 125I to a specific activity of 55 μCi/μg with chloramine-T (Diagnostic Systems, Webster, TX). A saturation curve was constructed for 100 μl volumes using 10 ng of 125I-labeled Tat and eight serial twofold dilutions of unlabeled Tat starting at 200 μg/ml. Solutions were added to non-tissue-culture-treated 96-well microtiter plate wells (#76-231-05, Flow Laboratories, McLean, VA) and incubated overnight at 4°C. Solutions were next replaced with 100 μl of 2.5% BSA and further incubated at 37°C for 2.5 hours. After removal of BSA, the wells were cut out with a hot wire device and the adsorbed label in each well was directly measured in a Compu-gamma gamma counter (Wallac, Inc., Gaithersburg, MD). The surface area of contact for 100 μl volume in the wells was calculated to be 0.9 cm². Results are the mean surface density of three replicate values.

**Fluorescent labeling**

Cells were labeled as described previously (Chan and Aruffo, 1993). Subconfluent HT1080 cells were lifted off the flask by gentle scraping, washed two times and suspended in serum-free MEM medium with Earle’s salts. Cell viability was assayed with trypan blue and found to be >95%. 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxy fluorescein, acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes Inc., Eugene, Oregon. A 10 mM stock solution of BCECF was prepared in DMSO. Cell suspensions at 10⁶ cells/ml were made 10 μM in BCECF with stock solution and incubated at 37°C for 30 minutes, with occasional mixing. Cells were washed three times with 10 ml of serum-free MEM to remove excess label and finally suspended at a concentration of 5×10⁵ cells/ml in the same medium containing 0.05% (w/v) BSA (Calbiochem, La Jolla, CA).

**Attachment/adhesion assay**

Test wells of Linbro non-tissue-culture-treated 96-well plates (#76-231-05, Flow Laboratories) were coated with 100 μl of various concentrations of Tat, FN, or VN diluted in PBS. Test wells and negative control wells without substrate were incubated overnight at 4°C. Before use, plates were rinsed two times with PBS and wells were blocked with 100 μl of 2.5% (w/v) BSA in PBS for 2-3 hours at 37°C to minimize non-specific binding. Plates were rinsed three times with PBS, 100 μl of the BCECF labeled cells (5×10⁵ cells/ml) were added to each well and the plates were incubated for one hour at 37°C in an atmosphere containing 5% CO₂ or at 4°C in a cold room. When adhesion was performed at 4°C, substrate-coated plates as well as cells were prechilled separately for 10 minutes.

Unattached cells were separated from the substrate bound cells by the application of buoyant force using Percoll™ induced density inversion. At the end of the attachment incubation at 37°C or 4°C, 200 μl of Percoll™ (Sigma, St Louis, MO) diluted 9:1 (v/v) with 10× PBS was added gently to the side of each well. The denser Percoll™ sinks to the bottom, lifting the unattached cells, along with medium, to the top of the wells. A clear interface between the Percoll™ and colored medium was maintained. The top fluid containing the unattached cells was removed by wicking with height-adjusted 4×12 Skatron harvesting frames (Skatron Inc., Sterling, VA), leaving the bottom 75 μl of fluid in the wells. This process was repeated once again with great care taken to avoid any disturbance of the attached cells. Percoll™ used for washing was maintained at the same temperature at which adhesion was performed. In our hands, when the Percoll™ was added just under the medium surface and in contact with the wall at a rate of less than 20 μl/second, only those cells attached with a force less than the buoyancy force were lifted. Visual observation confirmed that there was no shear-induced disruption of cell monolayer continuity due to the addition of Percoll™. Fluorescent label in the attached cells was measured at excitation/emission wavelengths of 485/535 nm using a Fluorescence Concentration Analyzer (Baxter Healthcare Corp., Mundelein, IL). The relative fluorescent intensity (RFI) increased linearly with increasing cell numbers (data not shown). However, day to day variations were observed in the absolute value of fluorescent intensity due to variations in the uptake of dye by the cells. Since the calibration was not done on each experimental day, we did not express detachment in cell numbers, but instead gave it as the mean of three replicate values of the RFI. Experiments were routinely monitored by fluorescence microscopy for visual confirmation that the cells were attached as well as to detect any evidence of adhesion by cell spreading.

We compared our method for removal of unattached cells to a more conventional PBS washing protocol (Ruoslahti et al., 1982). In the conventional method, following attachment incubation, the supernatant was aspirated and 200 μl of PBS was added gently with a multichannel pipette. This was repeated twice and the fluorescent label in the attached cells measured as described above.

The strength of cell-substrate attachment was measured by adding 200 μl of Percoll™ to the wells following the attachment incubation and centrifuging each plate in a Beckman GS-6 series centrifuge for 10 minutes at a single speed, 600, 1,200, 1,800, 2,400 or 2,750 rpm (68, 271, 609, 1,083 or 1,422 g, respectively). The temperature regulator on the centrifuge was set to the temperature at which adhesion was performed. At the end of centrifugation, unbound cells were removed with Skatron filters and the remaining fluorescence in attached cells was measured as described previously. The force (F) applied to each cell is (Archimede’s Theorem):

\[ F = (\rho_c - \rho_m) V_c R CF, \]

where \( \rho_c \) is the density of the cell, \( \rho_m \) is the density of the medium, \( V_c \) is the volume of the cell, and RCF is the relative centrifugal force.
The cell to substrate adhesive force must be greater than the applied force for the cell to remain attached. To determine the cell densities, gradients were produced in Percoll™ using density marker beads (Sigma) according to the manufacturer’s instructions. The density of HT1080 was 1.046 g/ml and the density of Percoll™ diluted with 10× PBS was 1.123 g/ml. The cell volume was determined using a Coulter counter and was found to be 1.455 nl. Therefore, the force exerted at unit gravity on a single HT1080 cell in Percoll™ is 0.112 μdynes.

RESULTS

We have measured the strength of attachment of HT1080 cells to Tat protein in comparison with the well-known adhesion molecules, FN and VN. Our initial attempt to measure cell adhesion to Tat in the 96-well microtiter plates involved removal of the unattached cells by washing with gentle pipetting and aspiration with PBS which resulted in very high background fluorescence values for both Tat-coated and negative control wells. When we did succeed in removing the cells from the negative control wells by more vigorous washing, this also removed cells from the substrate coated wells, and thus did not differentiate cell attachment to Tat from that of background binding. In order to apply a uniform, gentle and well-defined force, we displaced the normal medium with a denser Percoll™ solution. Table 1 shows that the density floatation significantly reduced the background binding of the cells, allowing a clear distinction of cell binding to Tat from that to BSA alone.

**Binding as a function of Tat, FN and VN coating concentration**

Using the density inversion protocol detailed in Materials and Methods, we investigated the binding of cells to Tat, FN and VN at both 4°C and 37°C as a function of the coating concentration, Cc (Fig. 1a,b). All cells bound by a force of less than 0.1 μdynes/cell were removed by the buoyant force. The protein coating concentration giving half maximum cell attachment (Cc 50) is the most important parameter to be extracted from concentration curves. This parameter is calculated from the detachment curves by finding the Cc associated with a fluorescence (RFI) reading of: {RFI(maximum for substrate)+RFI(control)}/2. Note that a lower value of this parameter indicates more efficient binding per molecule of substrate.

Although concentration dependent attachment of the cells was observed for all the substrates tested, saturation with respect to coated ligand concentration was reached for FN and VN but, not for Tat. Previous estimates of the saturation of cell binding with respect to Tat concentrations of ≥4 μM for HeLa cells and 0.5 μM for H9 cells (Mann and Frankel, 1991) show a similar lack of saturation. At both 4°C and 37°C, the Cc 50 for FN or VN was much lower than that of Tat. The Cc 50 at 4°C was 6 nM for FN, 18.7 nM for VN and ≥300 nM for Tat (Fig. 1a). The Cc 50 at 37°C for FN and VN was about 2 nM for both and ≥300 nM for Tat (Fig. 1b). Thus, the Cc 50 of the ECM molecules decreased with increasing temperature, while that for Tat did not.

At 37°C, HT1080 cells were spread on both FN and VN, but

**Table 1. Effect of washing conditions on HT1080 cell attachment to Tat peptide**

<table>
<thead>
<tr>
<th>Wash medium</th>
<th>Cells remaining†</th>
<th>Cells remaining†</th>
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</thead>
<tbody>
<tr>
<td>PBS</td>
<td>16119±1048</td>
<td>19884±4200</td>
</tr>
<tr>
<td>Percoll™</td>
<td>18134±339</td>
<td>24544±483</td>
</tr>
</tbody>
</table>

*HT1080 cells were labeled with BCECF, washed, incubated for attachment and fluorescence measured as described in Materials and Methods. Each data point represents the mean RFI ± s.d. from three replicate wells.
†Mean RFI ± s.d. from three replicate wells.

Fig. 1. (a) HT1080 attachment at 4°C as a function of substrate concentration. HT1080 cells were labeled with BCECF, washed, incubated for attachment and fluorescence measured as described in Materials and Methods. Each data point represents the mean RFI ± s.d. from three replicate wells. The single point for negative control binding indicates the background binding for no initial coating ligand and identical processing thereafter. (b) HT1080 cell attachment at 37°C as a function of substrate concentration. HT1080 cells were labeled with BCECF, washed, incubated for attachment and fluorescence measured as described in Materials and Methods. Each data point represents the mean RFI ± s.d. from three replicate wells. The single point for negative control binding indicates the background binding for no initial coating ligand and identical processing thereafter. Symbols defined in the legend as ‘spread’ are indicated starting with the lowest concentration for which cell spreading could be observed.
not Tat. Cell spreading was first noticed at 5 nM FN and 18.7 nM VN. The fraction of spread cells increased to 100% by 50 nM FN and 187 nM VN. Fig. 1b shows that the dose-response curve had already reached a maximum before any spreading was noticed.

**Strength of attachment determined by increasing buoyancy force**

The strength of attachment to Tat, FN and VN was investigated by directly centrifuging the upright microtiter plates after Percoll™ addition. In the previous section we found that the Cc for maximum cell attachment (i.e. the plateau) was dependent on the coated protein. Instead of using the same Cc for all substrates and temperatures, we decided to use the minimum Cc that showed maximum attachment. This allowed the raw fluorescence readings for all the substrates to be approximately equal at low force. Furthermore, it compensated to some degree for differences between spread and non-spread cells. The following Cc were chosen to study the strength of HT1080 cell attachment: at 4°C, 1,000 nM Tat, 15 nM FN and 56 nM VN; at 37°C, 300 nM Tat, 5 nM FN and 18.7 nM VN. The results are background subtracted (negative control wells were measured at each force) and then normalized by the maximum reading (Fig. 2a and b). The raw readings for the negative control wells are also shown. To facilitate comparison of the results, we define the force at which 50% of the cells remain attached as the half maximum force (F50). The results at unit gravity are those obtained from the Cc studies. Force values were derived from the centrifugal force as described in Materials and Methods using the values we measured for cell density (1.046 g/ml) and cell volume (1.455 nl).

The detachment force curves at 4°C of FN and VN are very similar. The highest possible force in our experiment was not enough to remove all the cells, with 20% of the cells still remaining attached to these proteins (Fig. 2a). A force of about 500 g, equivalent to 55 μdynes/cell, was required to dislodge 50% of the cells from the matrix proteins. The detachment force of cells bound to Tat was higher, about 40% cells still remained attached at the highest force exerted (160 μdynes/cell). The F50 on Tat was found to be about 145 μdynes/cell (Fig. 2a).

The detachment force curves at 37°C (Fig. 2b) were quite different from those at 4°C. Increasing the temperature increased the binding energy for FN and VN, but not for Tat. Cells bound to Tat at 37°C were easily removed, with a F50 of about 400 g (35 μdynes/cell). At about 600 g all the cells were completely dislodged from Tat. At this temperature, the FN and VN curves are essentially identical, and it appears that above about 1,000 g (130 μdynes/cell) a plateau is reached (20 to 40% of cells detached), with no further removal of cells at higher force.

**DISCUSSION**

HIV-1 Tat protein has been shown to mediate cell attachment to a variety of cell types via an uncharacterized mechanism. While integrin and non-integrin receptors in cell-Tat interaction have been implicated (Brake et al., 1990; Barilliari et al., 1993; Vogel et al., 1993; Weeks et al., 1993), Mann and Frankel (1991) have suggested an endocytotic mechanism for the entry of Tat. Cell binding to Tat at 37°C were easily removed, with a F50 of about 400 g (35 μdynes/cell). At about 600 g all the cells were completely dislodged from Tat. At this temperature, the FN and VN curves are essentially identical, and it appears that above about 1,000 g (130 μdynes/cell) a plateau is reached (20 to 40% of cells detached), with no further removal of cells at higher force.

**Fig. 2.** (a) Strength of cell attachment to Tat, FN and VN at 4°C. To increase the dynamic range of the experiment, we used the minimum Cc that showed maximum attachment for each substrate. HT1080 cells were labeled with BCECF, washed, incubated for attachment to Tat (1.000 nM), FN (15 nM) and VN (56 nM), and fluorescence measured as described in Materials and Methods. Each data point represents the mean RFI ± s.d. from three replicate wells. Background binding was determined for no initial coating ligand and identical processing thereafter. The background binding was subtracted and cell attachment to each of the substrates was normalized by the maximum fluorescence values which are: Tat-21817; FN-19265; VN-23244. Strength of attachment is proportional to the fraction of cells remaining attached to substrates as a function of force. (b) Strength of cell attachment to Tat, FN and VN at 37°C. HT1080 cells were labeled with BCECF, washed, incubated for attachment to Tat (300 nM), FN (5 nM) and VN (18.7 nM) and fluorescence measured as described in Materials and Methods. The selection mechanism for choosing the substrate coating concentrations was described in the legend for Fig. 2a. Each data point represents the mean RFI ± s.d. from three replicate wells. Background binding was determined for no initial coating ligand and identical processing thereafter. The background binding was subtracted and cell attachment to each of the substrates was normalized by the maximum fluorescence values which are: Tat-16390; FN-18067; VN-16627. Strength of attachment is proportional to the fraction of cells remaining attached to substrates as a function of force.
of Tat into HeLa cells, without the involvement of a receptor. Tat has been shown to stimulate the proliferation of the spindle shaped ‘precursors’ of Kaposi’s sarcoma (KS) cells (Barillari et al., 1992) which are frequently observed in patient’s lesions, and thus could play an important role in the development of KS. In an attempt to understand the significance of cell attachment to Tat and to determine whether Tat can function as an adhesion molecule, we have studied the attachment of fibrosarcoma (HT1080) cells to Tat adsorbed on polystyrene. In agreement with earlier reports, and our own unpublished data, we observed that HIV-1 Tat protein mediated significant attachment to a number of cell types. In this report we describe the interaction of Tat with only one cell type, fibrosarcoma cells (HT1080).

Methodology

Cell-substrate interactions involving weak forces are often undetected in routine washing procedures, due to the variable and non-reproducible shear forces generated. We have observed that removal by careful washing with aqueous buffer of cells unbound to Tat resulted in either excessive erosion of attached cells or very high background binding (if the irrigation flow rate was too slow) and thus did not differentiate between cell attachment to Tat from that to the control background (Table 1), further indicating that cell-Tat interactions involve weak forces. We therefore employed an assay using highly regulatable buoyant forces to separate unattached cells from cells attached to the substrates. Since no erosion of cells was observed at the well bottom surface directly below the point where the denser fluid was added (where the shear forces are greatest), we feel confident that shear forces due to the addition of Percoll™ (under the conditions stated) are negligible compared to the buoyancy force. Thus, the detachment force applied during the washing step is the buoyant force at 1 g. Cells remaining attached at 1 g are subsequently removed by the application of increasing centrifugal force. Currently, the detection of attachment forces lower than detected here are potentially compromised by the shear flow generated during the addition of Percoll™. The minimum buoyant force required for detachment could be lowered by further optimizing the rate of addition of the denser solution, the buoyant density of the medium, or both. On the other hand, the maximum applied force could be increased by increasing the buoyant density of the medium, the centrifugal force, or both.

With the help of this procedure, we were able to precisely study weaker or initial cell-substrate interaction forces such as those occurring at lower temperatures and lower substrate concentrations (McClay et al., 1981; Lotz et al., 1989). Previous techniques used to measure cell-substrate detachment forces generated in microtiter plate wells by centrifugation have required that the wells be filled with medium, sealed, and placed in the centrifuge in an ‘inverted’ position. At the end of the centrifugation, the plates had to be quickly-frozen to avoid settling of the detached cells, and then the cells that remained attached were isolated by cutting off the bottom of the well and counted radio-isotopically (McClay et al., 1981; Lotz et al., 1989). These experimental manipulations were extremely cumbersome and made avoidance of leakage very difficult. Recently, Goodwin and Pauli (1995) have published an assay using Percoll™ irrigation combined with glutaraldehyde fixation as a washing procedure to fix adherent cells in place while displacing cells more weakly attached than the buoyant force provided by the Percoll™ at unit gravity. In their study, biotin-labeled cells were detected by subsequent enzyme-linked avidin assay. Our study differs from theirs in two significant respects. First, we were able to utilize higher than unit gravity forces to extend the range of observations over a much wider field of interaction energies, and second, we used direct fluorescence labeling of cells, which greatly simplifies the post-adhesion measurements of cell number.

Experimental approaches

The relationship and distinction between the two binding studies performed, coating concentration dependence, and attachment force dependence, cannot be over-emphasized. By varying the Cc, we change the surface chemistry that the cell encounters (either by adding sites or by covering or uncovering binding regions on the molecules) and then determine the fraction of cells that resist a constant (albeit low) force. In the force dependence experiments, the surface chemistry is held constant, and we determine the fraction of cells with attachment energy greater than the applied force.

Concentration experiments

A number of major observations can be made from the results of Fig. 1a and b. First, the dependence of cell attachment on surface coating concentration and temperature are clearly different for Tat and FN or VN. Second, the coupling of adhesion events with cytoskeletal participation, as evidenced by observable cell spreading, is likewise different for Tat and FN or VN. Finally, cell attachment to FN or VN is far more efficient than to Tat at 4°C or 37°C. The cell attachment curves for Tat at 4°C and 37°C do not appear to be distinguishable and do not reach saturation with respect to coating concentration. This is consistent with the observations of Mann and Frankel (1991), who estimated >10⁷ Tat binding sites per cell for HeLa and H9 cells and who also were unable to demonstrate saturation binding of Tat. Finally, there was no observable cell spreading at any Tat concentration or temperature tested. On the other hand, cell attachment onto both FN and VN clearly reaches saturation at 4°C and 37°C (Fig. 1a,b), and both FN and VN display a reduction in the coating concentration requirement for attachment at 37°C (Fig. 1b). The latter result is especially interesting when the concentration dependence of the observed cell spreading on FN and VN is taken into account, since the cell attachment curve is nearly complete before any cell spreading has begun to appear (Fig. 1b). Thus, observations of cell binding interactions at surface ligand concentrations lower by at least one order of magnitude than normal physiological response concentrations are achievable by our procedure. Furthermore, it is interesting to speculate that the cell attachment interactions taking place at pre-spreading concentrations of ECM protein may also be similar to those involved in preliminary steps of cell spreading at physiological concentrations of surface ligand. Lotz et al. (1989) have previously suggested that cell attachment interactions at 4°C may similarly serve as initial attachments for subsequent strengthening responses at 37°C.

Force experiments

Most of the current theoretical analyses of receptor mediated bond strengths are based on the work of Bell (1978). Using an
approach based on the kinetic theory of isotropic materials, he proposed that the dissociation rate constant was a function of the applied stress. Using what were considered to be reasonable parameters, he found a force of 4 dynes per bond. The estimated strength of a receptor-ligand bond was nearly equal to the force necessary to pull a glycoporphin molecule through a lipid bilayer (Bell, 1978). It appears from Fig. 2a,b that if we use this bond strength, then the number of bonds per cell is low, in the order of 100. One of the goals of this paper was to be able to compare and distinguish, if possible, the energies of attachment of a variety of substrate molecules to a common reference, HT1080 cells. To achieve this goal, we will need to correctly normalize the results of the experiments performed. To do this we need to evaluate three parameters for each of the substrates. These parameters are: the surface density of the substrate molecules bound to the plastic, the surface density of receptors on the cell surface, and the area over which the adhesion takes place. We will now discuss the estimation of these variables.

**Ligand surface density parameter**

The analysis of the force data was made more difficult by the decision to use the minimum concentration that gave the maximum attachment (i.e. different values for each protein, as described in Results). Under these conditions it is important to stay within the linear regime (i.e. where the adsorption isotherm is a linear function of the $C_C$), so that we can be fairly sure that an increase in the $C_C$ will lead to a corresponding increase in the number and activity of molecules adsorbed on the surface. Therefore, the first variable we need to evaluate is the adsorption isotherm, the relation between the $C_C$ and the substrate surface density. For the ECM proteins, there are several available sources for the adsorption isotherm (DiMilla et al., 1992; Underwood et al., 1993). Underwood et al. (1993) measured adsorption to non-tissue-culture-treated polystyrene plates and found the upper limit of the linear adsorption isotherm to be at a coating concentration of about 5 µg/ml for FN and between 2.5 and 5 µg/ml for VN. They postulated that continued adsorption above the linear limit could be due to either a change in the orientation of FN and VN molecules from ‘side on’ to ‘end on’, or to multilayer adsorption. We calculated the adsorption isotherms for the ECM molecules from the data of Underwood et al. (1993) and present it in Table 2. The isotherm for Tat was determined as described in Materials and Methods and is also presented in Table 2. The range of Tat, FN and VN $C_C$’s we chose for the force studies are within the linear range of their isotherms.

**Cell contact area parameter**

The areas of close approach of the cell to a solid support have been studied (Lotz et al., 1989) but are difficult to obtain since the evanescent wave techniques needed to unequivocally determine separation are not commonly available and experimentally difficult to perform. Experiments in this laboratory to study these domains of close attachment using total interference reflection microscopy are now in progress. Lacking a direct measurement, the contact area is customarily taken to be the projected area of the cell that is within 50 nm of the surface, the approximate distance over which a receptor mediated bond can act (Mege et al., 1987). For a nonspread cell, modeling the HT1080 cell as a sphere of diameter 14 µm, the contact area is 2.2 µm$^2$. When the cell spreads, the total cell area as seen by gross morphology increases by a factor of two to three (Lotz et al., 1989; Truskey and Proulx, 1993). Since all of this area is available for adhesion, the upper limit of contact area would now be 200 times greater than in the nonspread case. However, we do not have accurate data on spreading, so the best normalization for our results will be the simple projected area (1/4πD$^2$). Using IRM measurements of close contact area between glioma cells and FN, Lotz et al. (1989) arrived at a value of ~125 for this ratio.

**Receptor density parameter**

The cell surface density of receptors might conceivably be estimated for FN and VN using anti-integrin mAb. Unfortunately, the nature of the cell surface molecules that interact with the Tat protein has been quite controversial. HT1080 cells possess specific integrin receptors that promote attachment to the RGD regions of the FN and VN and promote their spreading on these substrates. Weeks et al. (1993) suggested that the basic domain of Tat mediates attachment of H9 lymphocytes through a non-integrin receptor. Vogel et al. (1993) reported that an atypical integrin, $\alpha_5\beta_5$, on human leiomyosarcoma cell line (SK-LMS) binds to the highly basic domain, but not to the RGD sequence of Tat protein. On the contrary, involvement of $\alpha_5\beta_1$ and $\alpha_5\beta_3$ integrin receptor binding to Tat through its RGD sequence has been presented using human umbilical vein endothelial cells and AIDS-Kaposi sarcoma cells (Barillari et al., 1993). Because of the lack of consensus concerning Tat receptors, and the uncertainties introduced by

Table 2. Coating concentrations and equivalent surface densities of Tat, FN and VN*

<table>
<thead>
<tr>
<th></th>
<th>Tat</th>
<th>FN</th>
<th>VN</th>
</tr>
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<tbody>
<tr>
<td>Concentration</td>
<td>µg/ml</td>
<td>Surface density</td>
<td>Concentration</td>
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<td>10.0</td>
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<td>0.54</td>
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<td>0.66</td>
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<td></td>
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<td>0.22</td>
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*Surface density of Tat was determined as described in Materials and Methods. Densities of FN and VN were interpolated from the data of Underwood et al. (1993).

†The actual surface density of FN at the highest concentration coated, 22 µg/ml was extrapolated from the data of Underwood et al. (1993).
surface diffusion of receptors in the cell membrane, we were unable to address receptor density in our analysis. Therefore we were unable to interpret our results on a ligand-cell receptor bond basis and were restricted to an analysis based on surface ligand density and projected cell contact area.

**Data analysis**

We analyzed the data using a reduced force, $F^\text{red}$, the force divided by the number of coated substrate molecules in the calculated binding area (i.e. the centripetal force normalized by the number of potential bonds). We used surface densities of the surface coated molecules extracted from the adsorption isotherms along with the total projected area of the cell to obtain the number of molecules in the binding area. The fraction of cells remaining (after subtracting out the control binding) was then plotted against this reduced force (in dynes/picomole) (Fig. 3). These reduced force binding curves help us understand the binding of HT1080 cells to the three substrates (Fig. 3). These cells display the expected temperature dependence with FN and VN. The $F^\text{50}$ increases by an order of magnitude or more when the temperature is increased from 4°C to 37°C as expected for a physiological, in this case receptor-coupled, attachment event. The $F^\text{50}$ for Tat is the same at both temperatures, suggesting that it has a only a non-integrin attachment interaction with the cell, despite the presence of an RGD sequence. The absence of a temperature dependence of attachment energy with Tat is in agreement with our earlier unpublished observation that metabolic inhibition with azide did not affect cell attachment to Tat at 37°C. From experiments in progress with recombinant, transactivationally active Tat it appears the synthetic and recombinant materials behave similarly with respect to cell attachment. Thus cell attachment to Tat may not be highly dependent on its secondary or tertiary structure.

It is evident that HIV-1 Tat can engage in a significant but weak cell attachment, with a half-maximal force ($F^\text{50}$) in the order of 3.0 (dynes/picomole) at both 4°C and 37°C, and does not mimic typical adhesion molecule physiological binding functions. The strength of cell attachment to Tat is about two orders of magnitude lower than that seen to ECM molecules and confirms that Tat-mediated cell attachment is not likely to be integrin receptor driven. Nevertheless, there is evidence for the ability of HIV-1 Tat to enter cells (Frankel and Pabo, 1988; Mann and Frankel, 1991; Ensol et al., 1993) and in some cases may perturb the cell machinery growth regulation (Ensol et al., 1990), transcription (Buonaguro et al., 1992; Taylor et al., 1992; Opalenik et al., 1995), and signal transduction (Li et al., 1995; Westendorp et al., 1995). The absence of the evidence for attachment through a cytoskeleton-coupled receptor for Tat does not preclude other types of receptors including other classes of adhesion receptors which are not cytoskeleton-coupled such as glypicans and Contactin/F11, which bind to Tenascin and are anchored to the cell membrane via glycosphosphatidylinositol (Vaughan et al., 1994). We are currently investigating the relationship between the observed attachment forces and the mechanism through which Tat binds to and enters the cell.

**Conclusion**

In summary, we have used a buoyant density assay to demonstrate that HIV-1 Tat protein mediates HT1080 cell attachment both at physiological and non-physiological temperatures. However, Tat does not induce cell spreading at 37°C, unlike the ECM proteins FN and VN. A comparison between Tat and the ECM proteins was made in terms of strength of cell attachment. We found that Tat mediates a significant but weak cell attachment in the order of 3.0 (dynes/picomole) both at 4°C and 37°C, suggesting a non-integrin interaction of cells with this protein. ECM proteins, on the other hand, showed a significant temperature dependent strengthening of attachment. The procedures we have developed in this study are simple, rapid, and yield results which are based on quantifiable physical principles and may be interpreted on cellular and molecular grounds. Furthermore they require only a simple apparatus which is found in most laboratories, a commonly available table top centrifuge. We believe that this will allow these procedures to be readily and beneficially applied to a wide variety of cell-cell and cell-matrix interaction studies in the future.

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**REFERENCES**


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**Fig. 3.** Analysis of the strength of HT1080 cell attachment to Tat, FN and VN. Since the decision to use the minimum substrate concentration that gave the maximum attachment resulted in different coating concentrations for each protein, the force of cell attachment is normalized to the surface number density of the substrate, the determination of which has been described in the Discussion. We define a reduced force as the centripetal force divided by the surface density of the coated molecules in the binding area (in μdynes/picomole). This figure shows the fraction of HT1080 cells (from Fig. 2a and b) remaining attached as a function of the reduced force for Tat, FN and VN at 4°C (open symbols) and 37°C (closed symbols).
protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence. *Proc. Natl. Acad. Sci. USA* 90, 7941-7945.


