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Biochemical and Biophysical Research Communications 363 (2007) 178–182
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Interactions of cell penetrating peptide Tat with model membranes: A biophysical study

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Received 21 August 2007
Available online 4 September 2007

Abstract

The protein transduction domain of the HIV-1 transactivator of transcription, Tat ($Tat^{(48–60)}$), has been shown to transport P10, a cytotoxic peptide mimic of the cyclin dependent kinase inhibitor p21WAF1/CIP1, into the nucleus of cancerous cells and induce apoptosis. Here, monolayer studies were used to investigate the membrane interactions of $Tat^{(48–60)}$, P10 and the construct $Tat^{(48–60)}$P10. It was found that $Tat^{(48–60)}$ showed no significant surface activity but that both P10 and $Tat^{(48–60)}$P10, were highly surface active, inducing surface pressure changes of 9.7 and 8.9 mN m^{-1}, respectively, with DMPS monolayers. The comparison of $Tat^{(48–60)}$P10 and P10 surface interactions would be consistent with a hypothesis that the cargo attachment influences the capacity of the Tat-protein transduction domain to mediate transport across membranes either directly or *via* localisation of the construct at the membrane interface.

Keywords: Cell penetrating; Lipid monolayer; Isotherm; Tat peptide

Cell penetrating peptides (CPPs) are generally 5–40 amino acids in length, have the capacity to traverse cell membranes and are able to deliver conjugated cargoes to the nucleus or cytoplasm [1]. A frequently employed prototypic CPP is that derived from the HIV-1 Tat protein [2,3], which possesses a highly basic minimal transduction domain of approximately nine amino acids (RKKRR QRRR). This protein transduction domain (PTD) has been successfully utilized to transport oligonucleotides, peptides, and proteins into a broad range of cell types, with cargo uptake occurring rapidly [4,5]. Cargo transduction by Tat and by other CPPs is therefore an area of intensive investigation because of interest in the intracellular delivery of novel pharmacological agents which by themselves are cell-impermeable [6].

Whilst the transducing capability of this Tat-derived peptide is not in dispute, the mechanism of internalization of the Tat-PTD in the presence or absence of conjugated cargoes is a deeply contentious field, primarily as a consequence of concerns regarding possible artefacts associated with cell fixation (e.g. [7]). Extensive investigation has resulted in a number of significantly different pathways being proposed for Tat-protein PTD derived translocation including: clathrin-dependent endocytosis, lipid raft-dependent macropinocytosis, and the use of non-endocytic pathways such as direct movement through lipid bilayers [7–11]. For endocytic mechanisms, a role for polyanionic cell-surface molecules including glycosaminoglycans such as heparan sulphate has been mooted in a number of studies [12–15]. In contrast, evidence for direct (ATP- and receptor-independent) membrane translocation by peptides derived from the Tat-PTD is principally based on findings that transduction inhibition occurs at 4 °C or in the presence of energy poisons, i.e. in conditions that are incompatible with endocytosis [16,17] (and reviewed in [18,19]). Whilst direct mechanisms of translocation are necessarily expected to invoke membrane perturbation, pore formation for Tat based transduction although a formal possibility

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.08.162

is not generally supported experimentally because of the relative lack of toxicity associated with the use of Tat or the Tat-PTD as a CPP.

We recently reported on the ability of the Tat$^{(48–60)}$ sequence (GRKKRRQRRRPPQ), to induce apoptosis when conjugated to the proliferating cell nuclear antigen interacting protein (PIP) box consensus sequence (denoted 'P10'). This ability was particularly evident in cells originating from gliomas [20] and immunofluorescence was able to show that the conjugate co-localised with the target cognate partner, PCNA in the cell nucleus. We report here how such Tat-PTD containing peptides with and without cargo (Tat$^{(48–60)}$, P10, Tat$^{(48–60)}$P10, and Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10) interact with pure lipid monolayers. We find that whilst the un-conjugated Tat-protein PTD itself shows a weak lipid interaction and thus is unlikely to spontaneously insert into bilayers, the ability of the relatively hydrophobic conjugated cargo to associate with cell lipids confers a membrane associating capacity onto the Tat-protein PTD. We believe this finding has significant implications for the methodologies used to investigate Tat-membrane binding and for the understanding of Tat-protein PTD based cargo translocation.

Materials and methods

Reagents. The peptides Tat$^{(48–60)}$P10 (GRKKRRQRRRPPQRQ TSMTDFYHSKRRLIFS), Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10 (GRKKRRQRRR PPQRATSATDAYHSKRRLIFS), P10 (RQTSMTDFYHSKRRLIFS), and Tat$^{(48–60)}$ (GRKKRRQRRRPPQ) were synthesized by solid phase and reverse-phase HPLC purified, with masses confirmed by MALDI-TOF mass-spectrometric analysis and in one case (Tat$^{48–60}$) by N-terminal sequencing, as previously reported [20]. Buffers and solutions for monolayer experiments were prepared from Milli-Q water. 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine, sodium salt (DMPS) were from Alexis Biochemicals (Birmingham, UK). All other reagents were purchased from Sigma (UK).

Peptide surface activity. Surface tension was monitored by the Wilhelmy method using a paper plate (Whatman's Ch1) in conjunction with a microbalance and a custom made polytetraflouroethylene Langmuir trough (area 15 cm^2, Nima technology) as described by Brandenburg et al., [21]. Peptides at concentrations ranging from 1.0 to 18 μM were injected into a Tris buffer (10 mM, pH 7.5) subphase *via* a vertical hole drilled in the trough wall. Changes in surface pressure at the air/water interface were monitored for 1 h. The maximal values of these surface pressure changes were then plotted as a function of the peptide's final subphase concentration (Fig. 1).

Peptide monolayer. The ability of each peptide to spread on an aqueous surface and to form a stable monolayer was determined in a NIMA 601M Langmuir–Blodgett trough. The barriers of the Langmuir–Blodgett trough were adjusted to their maximum separation (surface area 78 cm^2) and this position maintained. The surface was then cleaned by aspiration to remove any impurities checking the isotherm each time. Once the surface was free from any impurities, each peptide (2.5 mM) in methanol was spread on to the subphase containing a known number of peptide molecules (7.23×10^{15} molecules) using a high precision Hamilton microsyringe and the monolayer was allowed to settle for 1 h to ensure the methanol had evaporated off. The resulting peptide monolayer was compressed using the moveable barriers at a rate of 0.0678 nm^2/molecule/min. All experiments were carried out at room temperature (274 K).

The surface compression modulus (C_s^{-1}) of monolayers was calculated from surface pressure (π) and area per molecule data (A) applying Eq. (1).

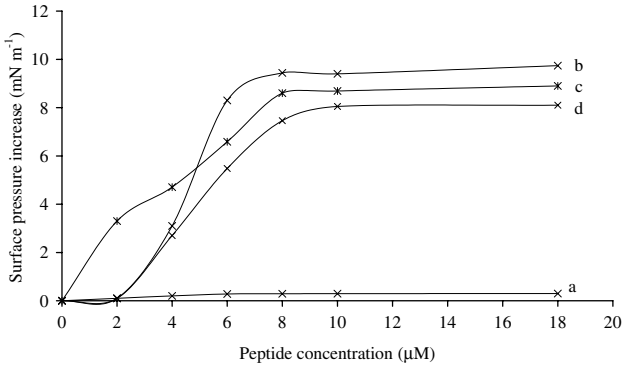

Fig. 1. Peptide surface pressure as a function of peptide concentration for peptides Tat$^{(48–60)}$ (curve a) P10 (curve b), Tat$^{(48–60)}$P10 (curve c), and Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10 (curve d).

$$C_s^{-1} = -A(d\pi/dA). \quad (1)$$

Lipid monolayers. Monolayer studies were conducted using Langmuir–Blodgett equipment supplied by NIMA Technology (Coventry, UK) interfaced with a PC and NIMA software thereby enabling a graphic output. Studies were conducted using a specially constructed 5 × 5 cm Teflon trough containing 15 ml of buffer subphase (10 mM Tris buffer, pH 7.5) which was constantly stirred at 15 rpm by a magnetic bar. Surface tension was monitored by the Wilhelmy method using a paper plate and a microbalance [22]. A Milli-Q water isotherm was recorded before each experiment to confirm the absence of surface impurities.

Monolayers were prepared from pure phospholipids, DMPC and DMPS, in chloroform solutions (5 mM), which were spread onto the air/buffer interface at an initial surface pressure of 30 mN m^{-1}, mimetic of biological cell membranes [23,24]. After evaporation of the chloroform and stabilisation of the pressure, for 120 s, the peptides (2 mM) in 25 mM Tris, pH 7.5, were injected into the subphase to achieve a final concentration of 10 μM. To ensure homogenous distribution of peptide in the subphase, the magnetic stirrer was set to a speed of 15 rpm. Peptide monolayer interactions were recorded as changes in monolayer surface pressure and the resultant data were stored on a computer. All of these experiments were repeated at least three times to ensure consistency.

Results

Peptide adsorption at the air/water interface

The ability of P10 and the Tat$^{(48–60)}$P10 constructs to absorb onto the air/water interface was determined for 1–10 μM solutions in the absence of lipid. The surface activity (Fig. 1) followed similar kinetics, clearly showing that at 8 μM saturation was achieved for P10, Tat$^{(48–60)}$P10, and Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10. All three peptides showed surface activity inducing surface pressure changes of 9.7, 8.9, and 8.1 mN m^{-1}, respectively. However, in contrast the Tat$^{(48–60)}$ peptide alone showed no significant surface activity which is in agreement with the weak amphiphilic character of the Tat sequence (Fig. 1).

To evaluate the surface activity for P10, Tat$^{(48–60)}$P10 and Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10, the surface excess, Γ, was calculated by means of the Gibbs' adsorption isotherm, which is given by the following equation [25]:

$$\Gamma = -\frac{1}{RT}\frac{\Delta\pi}{\Delta \ln c} \quad (2)$$

where R is 8.314 J mol^{-1} K^{-1}, $T = 294$ K, π is the interfacial pressure increase (mN m^{-1}) and c is the molar concentration of peptide in the subphase. These values of Γ were then used to determine area per peptide molecule at the interface by applying the following equation:

$$A = \frac{1}{N\Gamma} \quad (3)$$

where N is Avogadro's number (Table 1).

Table 1 shows that the calculated area per peptide molecule (A) decreased with increased peptide concentration at fixed pressure.

In order to verify the ability of the peptide to form stable monolayers, 7.23×10^{15} molecules of peptide were spread onto a Tris buffer interface and compression isotherms were undertaken. Fig. 2 shows each of the peptides P10, Tat$^{(48–60)}$P10, and Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10 were able to form stable monolayers at the air/water interface. The Tat$^{(48–60)}$P10 peptide is the least hydrophobic, which is supported by the lower maximum compression pressure (20.04 mN m^{-1}) and lower area lift off when compared to other two peptides. Peptide behaviour at an interface has been well documented [26] and it is thought that α-helical and β-sheet peptides can be distinguished by taking into account the extrapolated area of an isotherm. Fig. 2 shows the values of extrapolated area at $\pi = 0$ mN m^{-1} which is in agreement with those obtained for other peptides [26] and the calculated area/peptide molecule found at Table 1. These extrapolated values are in agreement with β-sheet formation (0.18–0.25 nm^2) in the case of P10 and the two constructs. To further analyse the physical state of the peptide monolayer, the compressibility modulus was calculated using Fig. 2. Davies and Rideal [27] indicate for peptide phases the compressibility modulus ranged from 1 to 20 mN m^{-1} which supported the compressibility values determined for our data (data not shown).

Interaction of the peptide with lipid monolayers

We investigated the mechanism by which the peptides might interact with membranes, by testing the ability of each peptide to penetrate lipid monolayers of constant area. Monolayers of either DMPS and DMPC were spread on to a buffer subphase at an initial surface pressure of

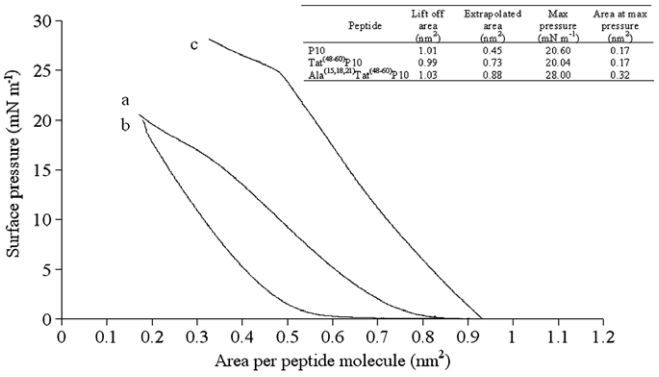

Fig. 2. A pressure-area isotherm for P10 (curve a), Tat$^{(48–60)}$P10 (curve b), and Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10 (curve c) monolayers, which were spread from chloroform onto a subphase of 10 mM Tris, pH 7.5.

30 mN m^{-1}, which was taken to represent that of naturally occurring membranes [23,24,28,29]. The peptides were then introduced into the subphase to give a final peptide concentration of 8 μM and the interactions of the peptide with lipid monolayers were monitored (Fig. 3A and B). It can be seen from Fig. 3 that for each monolayer studied, the insertion of each peptide followed parabolic kinetics that led to maximal surface pressure changes. In the case of P10, plus the Tat$^{(48–60)}$P10, and Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10 constructs the level of interaction is indicative of disruption of the monolayer acyl chain region by the peptides. These high surface pressure changes are comparable to those reported for other strongly membrane invasive peptides [30–32].

For DMPS monolayers (Fig. 3A), the peptides P10, Tat$^{(48–60)}$P10, and Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10 were found to rapidly partition the membrane over a period of 50 s inducing large maximal surface pressure changes of *circa* 9, 8, and 7.5 mN m^{-1}, respectively. The interaction of P10, Tat$^{(48–60)}$P10, and Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10 with the zwitterionic lipid, DMPC, showed a marked decrease in surface pressure as compared to its interaction with anionic DMPS inducing maximal surface pressure changes of *circa* 6, 5.4, and 5.3 mN m^{-1}, respectively. This decrease in the absence of the negative charge would imply electrostatic contribution from anionic species can stabilise membrane binding.

In contrast the Tat$^{(48–60)}$ peptide induced lower surface pressure changes of *circa* 2 mN m^{-1} which is indicative of headgroup interaction. Furthermore, whilst there was some reduction in pressure changes in the presence of DMPC as

Table 1
Excess, Γ (mol/m^2) and molecular area, A (nm^2) for each peptide at different concentrations assayed

Concentration (μM)	P10			Tat$^{(48–60)}$P10			Ala$^{(15,18,21)}$Tat$^{(48–60)}$P10		
	Δπ	Γ (×10^{-6})	A	Δπ	Γ (×10^{-6})	A	Δπ	Γ (×10^{-6})	A
4	3.1	2.30	0.72	4.7	3.48	0.48	2.7	2.00	0.83
6	8.3	6.60	0.25	6.6	5.26	0.32	5.5	4.37	0.38
8	9.4	7.90	0.21	8.6	7.28	0.23	7.5	6.31	0.26
10	9.4	8.30	0.20	8.7	7.73	0.21	8.0	7.15	0.23
18	9.7	9.90	0.17	8.9	9.05	0.18	8.1	8.24	0.20

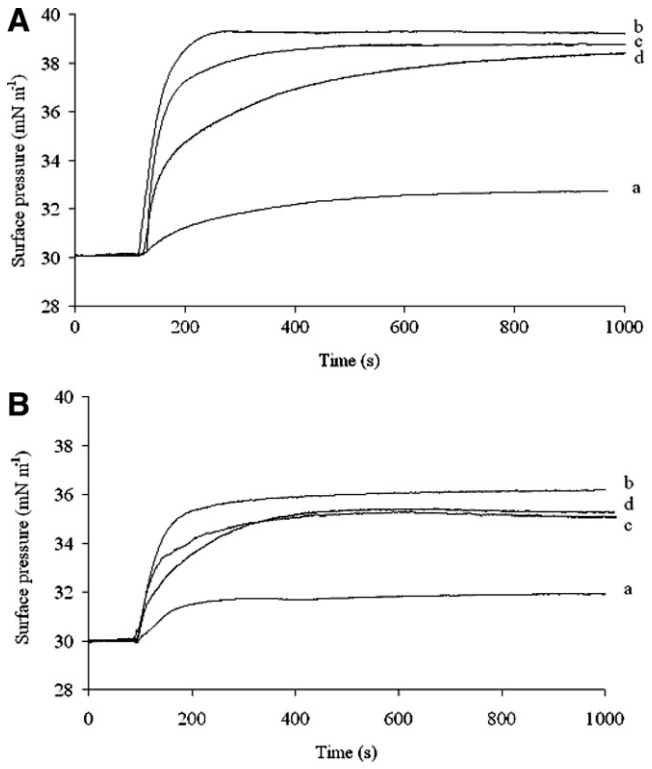

Fig. 3. The peptides for $Tat^{(48–60)}$P10 (curve a), P10 (curve b), and $Ala^{(15,18,21)}Tat^{(48–60)}$P10 (curve c) were found to rapidly partition into DMPS (A) and DMPC (B). However, $Tat^{(48–60)}$ (curve a) induced lower surface pressure changes of *circa* 1.5 mN m^{-1} for both DMPS (A) and DMPC (B) monolayers.

compared to DMPS this was not significant, which given the highly charged nature of the peptide emphasises further its relatively weak interactions at the monolayer interface.

Discussion

In light of the controversy surrounding Tat-protein PTD binding and internalization, we felt that a biophysical analysis of the $Tat^{(48–60)}$ and $Tat^{(48–60)}$—cargoes, binding to lipid monolayers would give insight to the process of Tat targeting and translocation. The limitations of such model systems have previously been noted, for example, there is a lack of an electrical gradient typically seen in cells, yet it has been suggested guanidium rich molecular transporters (such as Tat) may be translocated in a voltage dependent adaptive translocation mechanism [33]. However, the value of monolayer studies in investigating the important role of peptide/lipid interactions is well recognised [26,30,34]. Our findings indicate that whilst surface pressure changes induced by the 'naked' Tat-PTD were low (2 mN m^{-1}), the conjugates induced markedly larger surface pressure changes. This would fit with their amino acid composition which in the case of $Tat^{(48–60)}$ shows relatively low hydrophobicity and in either α or β structures would provide limited amphiphilicity therefore emphasising solubility and generating little partitioning at the interface. This is supported by the lack of surface activity shown by the peptide (Fig. 1).

In the case of the P10 and $Tat^{(48–60)}$P10 fusions the monolayer perturbations noted are of a magnitude consistent with previous reports of CPP surface effects (e.g. >15 mN m^{-1}; e.g. see [35]). It would thus appear reasonable to suggest that for this case the cargo contributes significantly to the capacity of the Tat-PTD construct to interact with the membrane. This would agree with a recent study using fluorescence spectroscopy to investigate Tat-PTD (residues 47–60), which concluded that there was no significant disruption or perturbation of the lipid bilayer by Tat alone [36].

Whilst research undertaken by Sabatier et al., [4] showed that $Tat^{(47–72)}$ interacted strongly with dilaurylphosphatidylserine membranes indicating a higher surface pressure increase than our reported results this may be due to the increased hydrophobicity in the amino acid residues 47–72. This would support our theory that the cargo (61–72 amino acids) affects the ability of the construct to partition at a membrane interface. Indeed, the only direct evidence for membrane perturbation by Tat involves generating more hydrophobic moeties. For example Tat has been shown to promote phospholipid vesicle fusion *in vitro* [17] but in that case tryptophan containing analogues of $Tat^{(48–60)}$ (TatP59W and TatLysP59W) were designed to increase the hydrophobicity of the helix [17]. In this latter case these manipulations of the sequence also increased the amphiphilicity of the peptide, which would be predicted to enable the peptide to penetrate the membrane at a shallow angle, thereby destabilising membrane lipid organisation and promoting fusion of the peptide into the membrane [37].

The ability of the cargo to enhance lipid penetration could coincidently aid localisation at the membrane interface in a manner comparable to that employed by some targeting signals [6]. This could support direct translocation or more likely support membrane by the construct thereby focusing diffusion within the plane of the membrane and increasing the efficacy of targeting the membrane translocating machinery. Interestingly the Tat constructs also showed a dependency for polyanionic surfaces which would support previous work exploring the role of such molecules [12–15] but again in contrast to the Tat above would support a key role for the cargoes. In summary, our findings support the contention that Tat-PTD-cargo membrane monolayer interaction is significantly different to that for a 'naked' Tat-PTD and thus provide indirect support for the notion that uptake for a 'naked' CPP may be rather different than for conjugated CPPs [1]. In addition to work on uptake pathways this has relevance not only to $Tat^{(48–60)}$P10 targeting but analyses where penetration of Tat in cells has been traced as partitioning into membranes could conceivably be affected when hydrophobic molecules (such as fluorescein) are attached to the Tat-PTD.

Acknowledgments

We sincerely acknowledge the help of Keith Holding and John Howl (University of Wolverhampton).

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