View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Available online at www.sciencedirect.com

ScienceDirect

Biochimica et Biophysica Acta 1768 (2007) 419–426

Biochimica et Biophysica Acta BBA

www.elsevier.com/locate/bbamem

Translocation and nuclear accumulation of monomer and dimer of HIV-1 Tat basic domain in triticale mesophyll protoplasts

Archana Chugh *, François Eudes

Lethbridge Research Centre, Agriculture and Agri-Food Canada, P.O. Box 3000, 5403-1st Avenue South, Lethbridge, Alberta, Canada T1J 4B1

Received 5 July 2006; received in revised form 22 November 2006; accepted 27 November 2006
Available online 30 November 2006

Abstract

Cellular internalization of cell-penetrating peptide HIV-1 Tat basic domain (RKKRRQRRR) was studied in Triticale cv AC Alta mesophyll protoplasts. Fluorescently labeled monomer (Tat) and dimer (Tat_2) of Tat basic domain efficiently translocated through the plasma membrane of mesophyll protoplast and showed distinct nuclear accumulation within 10 min of incubation. Substitution of first arginine residue with alanine in Tat basic domain (M-Tat) severely reduced cellular uptake of the peptide (3.8 times less than Tat). Tat_2 showed greater cellular internalization than Tat (1.6 times higher). However, characteristics of cellular uptake remained same for Tat and Tat_2. Cellular internalization of Tat and Tat_2 was concentration dependent and non-saturable whereas no significant change in cellular uptake was observed even at higher concentrations of M-Tat. Low temperature (4 °C) remarkably increased cellular internalization of Tat as well as Tat_2 but M-Tat showed no enhanced uptake. Viability test showed that peptide treatment had no cytotoxic effect on protoplasts further indicating involvement of a common mechanism of peptide uptake at all the temperatures. Endocytic inhibitors nocodazole (10 μM), chloroquine (100 μM) and sodium azide (5 mM) did not show any significant inhibitory effect on cellular internalization of either Tat or Tat_2. These results along with stimulated cellular uptake at low temperature indicate that Tat peptide is internalized in the plant protoplasts in a non-endocytic and energy-independent manner. Competition experiments showed that non-labeled peptide did not inhibit or alter nuclear accumulation of fluorescent Tat or Tat_2 suggesting active transport to the nucleus was not involved. Studies in mesophyll protoplasts show that internalization pattern of Tat peptide is apparently similar to that observed in mammalian cell lines.
Crown Copyright © 2006 Published by Elsevier B.V. All rights reserved.

Keywords: HIV-1 Tat basic domain; Cellular internalization; Plants; Triticale; Mesophyll protoplast

1. Introduction

HIV-coded Tat regulatory nuclear protein is 86 amino acids long and plays an important role in virus replication by binding to the viral TAR region to transactivate the viral promoter [1,2,3]. Frankel and Green independently demonstrated cellular uptake of the purified full length Tat protein by mammalian cell types when added to the surrounding media [4,5]. These studies were the first indication of cell permeation properties of Tat protein. Further studies on Tat-derived peptides revealed that the translocation activity of Tat regulatory protein resides in its nonapeptide basic domain Tat $_{49–57}$, RKKRRQRRR [6–8]. Tat basic domain is one of the shortest known cell-penetrating peptide that can independently translocate across plasma membrane with predominant accumulation in the nucleus especially in the nucleolar region [6]. The number of arginine residues is the determinant of the translocation ability of Tat basic domain. Deletion of an arginine residue or substitution with an alanine residue results in significant reduction of cell penetration of Tat basic domain in human cultured cells [9]. Tat is among the most extensively studied cell-penetrating peptide for human gene therapy (44% cell-penetrating peptide studies pertain to Tat) [10]. Tat and its oligomers can traverse plasma membrane with the cargo complex carrying macromolecules of much higher mass and size than their own. These cargo macromolecular components (proteins, drugs or DNA) cannot surpass

Abbreviations: CPW, cell protoplast wash; cv, cultivar; dsRNA, double stranded RNA; EDTA, ethylenediaminetetracetate; FDA, Fluorescein diacetate; FITC, fluorescein isothiocyanate; HPLC, high performance liquid chromatography; MS, Murashige and Skoog; M-Tat, Mutated Tat; PTD, protein transduction domain; RT, room temperature; ssDNA, single stranded DNA; TAR, transactivation responsive region; t-Boc, *tert*-butyloxycarbonyl

* Corresponding author. Tel.: +1 403 317 3358; fax: +1 403 382 3156.
E-mail address: chuga@agr.gc.ca (A. Chugh).

0005-2736/$ - see front matter. Crown Copyright © 2006 Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2006.11.012

the entry barrier posed by cell membrane via conventional transport mechanisms. Versatility of Tat basic domain to interact with different molecules has made it an attractive tool for developing novel strategies for cellular delivery, thereby, adding new dimensions to human therapeutics and diagnostics [11]. Transduction and subcellular localization of various proteins (such as galactosidase, horse radish peroxidase, GFP) by fusion with Tat basic domain or linkage by streptavidin/biotin conjugation has been reported in different mammalian cell types [10,12–16]. The delivered proteins maintain their functionality and are biologically active in the cells. Tat can also mediate delivery of oligonucleotides [17], liposomes [18] and plasmid [19]. Branched version of Tat (8Tat) and the factors controlling the efficiency of Tat-mediated plasmid transfer have been studied [20,21]. Tat oligomers can also effectively translocate peptide–plasmid complex and exhibit enhanced gene expression in the presence of standard cationic transfection agents [22,23]. However, despite wide application of Tat basic domain in *in vitro* and *in vivo* studies in a range of mammalian cell lines, mechanism of its translocation across the biomembranes remains elusive and controversial by large. A number of reports suggest that cellular internalization of Tat occurs by free means, it is non-receptor mediated, endocytosis-independent but concentration-dependent [6,24]. Contradictory reports on involvement of endocytosis and membrane surface receptors such as heparan sulphate proteoglycans in translocation of Tat-mediated cellular delivery have appeared [22,23,25–28].

Interestingly, investigations on cellular internalization, cargo delivery by Tat and other cell-penetrating peptides have been mainly carried in the mammalian cell lines. In plants, peptide-mediated cellular delivery is a relatively nascent field. Cellular internalization of cell-penetrating peptides such as pVEC, transportan and penetratin has been recently reported in tobacco protoplasts derived from suspension cell culture [29]. Cellular uptake of arginine rich intercellular domain (AID) fusion proteins has been shown in onion epidermal and root tip cells, and tomato root cells [30]. Synthetic cationic homoarginine (R12) oligopeptide has been reported to deliver dsRNA into tobacco cells to induce post-transcriptional gene silencing [31]. However, cellular uptake of Tat basic transduction domain has not been characterized in plant cells. Keeping in view, the tremendous potential of cell-penetrating peptides in plant biotechnology, the present study has been undertaken to investigate cellular internalization of monomer (Tat) and dimer (Tat_2) of Tat basic domain in triticale mesophyll protoplasts. Triticale is a man made cereal (wheat × rye) with commercial and biotechnological value. Mesophyll protoplasts are a convenient system to investigate as they can be isolated in abundance with relative ease from leaves than protoplasts from any other plant tissue. Fluorescence microscopy and fluorimetric analysis were used to investigate translocation of fluorescently labeled Tat and Tat_2 across the plasma membrane of protoplasts. M-Tat served as negative control along with FITC-dextran sulphate. Influence of concentration, temperature, endocytic inhibitor(s) along with competition experiments with non-labeled peptide were undertaken to characterize cellular uptake of Tat peptides in mesophyll protoplasts.

2. Materials and methods

2.1. Mesophyll protoplast isolation and purification

Protoplast isolation and purification was carried out under aseptic conditions. Embryonal halves of the seeds of Triticale cv AC Alta were surface sterilized with 4% hypochlorite and inoculated on basal MS medium, pH 5.82 [32]. Peeled leaves from six-day-old seedlings were incubated in enzyme solution [2% cellulase and 2% macerozyme (Yakult Honsha Co Ltd., Japan) CPW solution, pH 5.6] for 4 h in dark, 25 °C. Protoplasts were isolated by centrifugation at 100×*g*, 3 min, RT (Eppendorf centrifuge 5810R, USA) washed twice with CPW solution and purified by layering on 21% sucrose in CPW solution. A band of protoplasts formed at the interphase was carefully removed and suspended in CPW solution. After two washings with CPW solution, protoplast density was adjusted to 10^6 protoplasts/ml.

2.2. Peptides synthesis and fluorophore labeling

Tat (RKKRRQRRR), Tat_2 (RKKRRQRRRRKKRRQRRR) and M-Tat (AKKRRQRRR) were custom synthesized at the Alberta Peptide Institute, University of Alberta, Canada. In brief, peptides were synthesized by solid-phase synthesis (model 430A, Applied Biosytems, Canada) using t-Boc strategy as reported by Hodges et al. [33]. For fluorophore labeling, carboxyfluorescein was added to the N-terminus of protected peptide before cleavage. Peptide cleavage from the resin was carried out with hydrofluoric acid. The product was purified on Zorbax 300SB-C8 reverse phase column. Fractions were identified by analytical HPLC (HP1100) on Discovery C8 column. Pure fractions were pooled and freeze dried. The purified product was hydrolyzed with HCl and analyzed on a Beckman 6300 amino acid analyzer using System Gold. Peptide molecular weight was confirmed using MALDI-TOF (Voyager DE Pro) mass spectrometry.

2.3. Incubation of protoplasts with fluorescently labeled Tat peptides

Mesophyll protoplasts (500 µl of 10^6/ml) were incubated with 400 µl fluoresceinated Tat, Tat_2 or M-Tat (5 µM) for 1 h in dark, RT, followed by washings with CPW solution. Protoplasts were then treated with Trypsin-EDTA (0.25%, Sigma-Aldrich):CPW solution (1:4) for 5 min followed by washings and final suspension in 500 µl CPW solution. The parameters remained constant for all the experiments unless otherwise mentioned.

2.4. Fluorescence microscopy

Protoplasts were observed under the fluorescence microscope (Axioskop 2 Mot Plus, Zeiss Inc, Germany) for cellular uptake and nuclear localization of fluoresceinated Tat and Tat_2 (Ex 490 nm /Em 520 nm). The percentage of protoplasts showing fluorescence was calculated using hemocytometer.

2.5. Fluorimetric analysis

The amount of fluorescence internalized by the protoplasts was detected using fluorimeter (Biorad, Versafluor, USA; Ex 490 nm /Em 520 nm). Protoplasts were lysed with 200 µl of 1% TritonX-100 (prepared in CPW solution) for 20 min, 4 °C. Besides treatment with M-Tat, set of negative controls comprised of protoplasts without any treatment and treated with FITC-dextran sulphate (Sigma Aldrich, 4000 kDa). Fluorimetric data presented is relative fluorescence uptake detected for each treatment.

2.6. Effect of different factors and inhibitors on cellular internalization of Tat and Tat_2

Concentration—Protoplasts were incubated with varying concentrations of fluoresceinated Tat, Tat_2 or M-Tat (0–30 µM). For each concentration 400 µl of the peptide was added with all the other parameters remaining the same as described in Section 2.3.

Temperature—Protoplasts were incubated with fluoresceinated Tat, Tat_2 or M-Tat at different temperatures (4 °C–37 °C) for 1 h in dark. Prior to incubation

with the peptides, protoplasts were pre-incubated at the respective temperatures for 20 min.

FDA staining test for protoplast viability—Viability of the protoplasts was tested after peptide treatment at different temperatures (4 °C–37 °C) using FDA staining method. A control with no peptide treatment was maintained at every temperature. Immediately after treatment, protoplasts (100 μl of 10^6/ml density) were incubated with 3 μl of 100 ng /ml FDA stain for 5 min in dark, RT. For the viability test non-labeled Tat or Tat_2 were employed to avoid interference by fluorescein of the labeled peptide with FDA under the fluorescence microscope. Viable protoplasts, that is fluorescing protoplasts were counted using hemocytometer.

Endocytic inhibitors—Protoplasts were pre-incubated with endocytic inhibitors [nocodazole (10 μM), chloroquine (100 μM), sodium azide (5 mM)] for 30 min, RT, followed by addition of fluoresceinated Tat or Tat_2.

2.7. Competition experiments

Competition experiments comprised of two sets: In the first set, the final concentration of the peptide added was fixed (5 μM). Various ratios of labeled and non-labeled peptide were employed (5:0, 4:1, 3:2, 2:3, 1:4, 0:5). In another set, a mixture of fixed concentration of fluoresceinated peptide (5 μM) with 5, 7.5, 10, 15, 20 μM of non-labeled peptide was added to the protoplasts. After incubation for 1 h, RT, protoplasts were washed with CPW solution followed by trypsin treatment and observed under fluorescence microscope or used for fluorimetric analysis.

3. Results

Potential for translocation of fluorescently labeled monomer and dimer of HIV-1 Tat basic domain was investigated in triticale mesophyll protoplasts. Influence of various factors such as peptide concentration, temperature during peptide treatment, addition of endocytic inhibitors on the cellular uptake of cell-penetrating peptides was studied. Competition experiments with non-labeled peptide were also carried to rule out active nuclear transport of Tat peptides.

3.1. Translocation and nuclear accumulation of fluoresceinated Tat and Tat_2 in triticale mesophyll protoplasts

Fluorescently labeled Tat and Tat_2 were efficiently translocated across the plasma membrane of mesophyll protoplasts and showed distinct nuclear accumulation. Protoplasts treated with M-Tat exhibited negligible fluorescence under the microscope. Also, fluorescence was not observed in the other two negative controls; untreated protoplasts and treated with FITC-dextran sulphate (Fig. 1A–D). The percent of protoplasts that showed fluorescence upon incubation with fluoresceinated Tat and Tat_2 was 20% and 23.5%, respectively (Fig. 2A). Sometimes, in case of Tat_2, fluorescence was also observed in the cytoplasm but not in a significant number of protoplasts compared to the distinct nuclear accumulation (data not shown).

Detection of cellular internalization of fluoresceinated Tat and Tat_2 was also carried out using fluorimeter. Tat_2 showed 1.6 times higher cellular translocation than Tat where as protoplasts incubated with M-Tat showed 3.8 and 6.3 times less fluorescence than Tat and Tat_2, respectively. Fluorescence was detected neither in the protoplasts treated with FITC-dextran sulphate nor in the untreated protoplasts (Fig. 2B).

3.2. Effect of concentration and temperature on cellular internalization of Tat peptides in triticale mesophyll protoplasts

Fluorimetric analysis revealed concentration dependent increase in the cellular internalization of fluoresceinated Tat and Tat_2. The two peptides showed similar pattern of concentration curve. Besides, the cellular uptake was non-saturable as the amount of fluorescence increased with the increase in concentration (0–30 μM; Fig. 3). Notably, M-Tat did not show

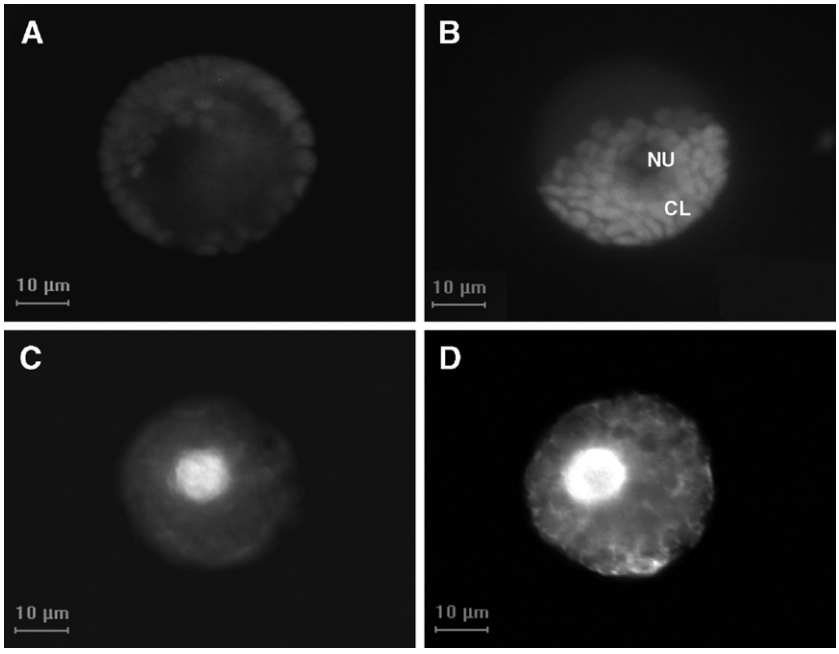

Fig. 1. Visual fluorescence microscopy showing translocation across cell membrane and nuclear accumulation of fluorescently labeled monomer (Tat) and dimer (Tat_2) of HIV-1 Tat basic domain in triticale mesophyll protoplasts. The protoplasts were incubated with 5 μM fluoresceinated cell-penetrating peptides in CPW solution for 1 h, RT. (A) control; (B) M-Tat; (C) Tat; (D) Tat_2. (Nu, Nucleus; Cl, Chloroplast).

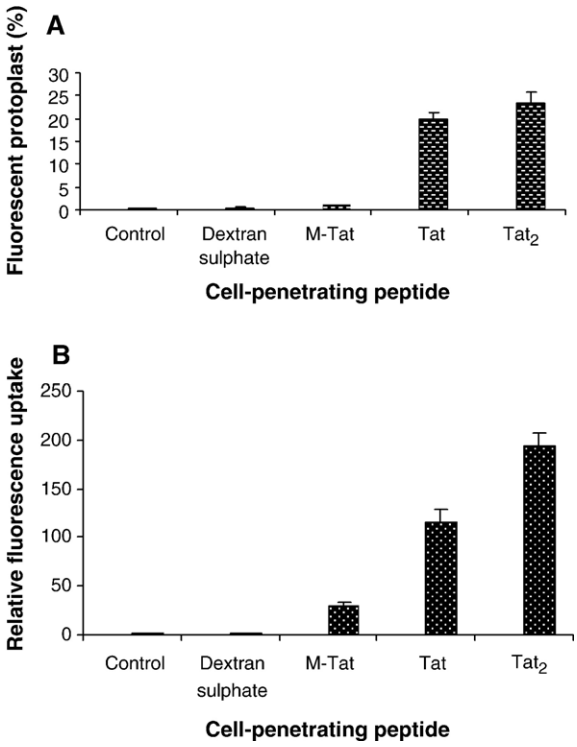

Fig. 2. Comparative cellular uptake of Tat peptides in triticale mesophyll protoplasts as observed under fluorescence microscope and by fluorimetric analysis. The protoplasts were incubated with 5 μM fluoresceinated cell-penetrating peptides in CPW solution for 1 h, RT. (A) (%) Fluorescent protoplast. (B) Relative fluorescence uptake.

significant increase in cellular uptake with the increase in concentration.

Translocation of both Tat and Tat_2 increased remarkably at low temperature (4 °C) as detected by fluorimetric analysis (1.6 times higher than RT). On the other hand, there was 1.5 times reduction in fluorescence uptake at higher temperature (37 °C). However, temperature range of 15 °C–28 °C did not have any significant influence on translocation of the peptides (Fig. 4A). Interestingly, at all the temperatures, the protoplasts showed nuclear accumulation of fluorescently labeled peptides. Low temperature did not enhance the cellular uptake of M-Tat as negligible fluorescence uptake was detected in comparison to Tat and Tat_2 (Fig. 4A).

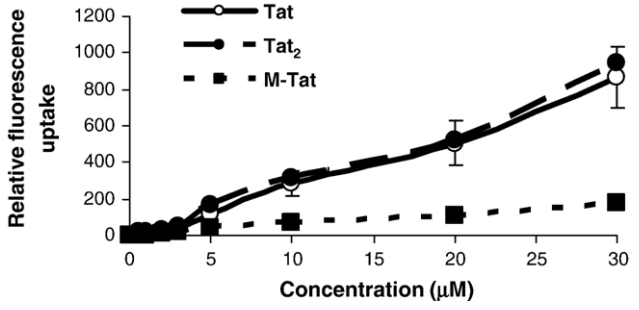

Fig. 3. Fluorimetric studies of the effect of concentration on the permeation potential of fluorescently labeled Tat peptides; Tat, Tat_2 and M-Tat in triticale mesophyll protoplasts.

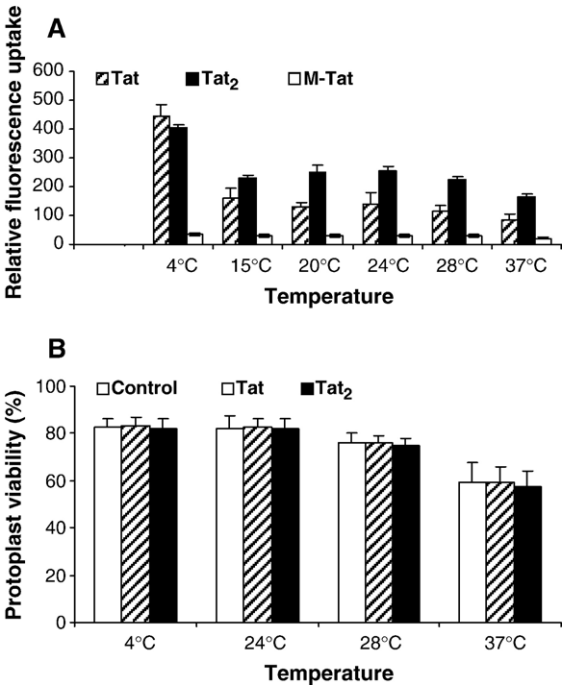

Fig. 4. (A) Effect of temperature on cellular internalization of the Tat peptides. (B) Viability test for protoplasts upon treatment with Tat peptides at different temperatures.

Protoplast viability was unaffected by the peptide treatment at temperature range 4 °C–24 °C, however, loss of viability was observed at higher temperature (37 °C) for the control as well as the treated samples (Fig. 4B).

3.3. Influence of inhibitors on cellular internalization of Tat peptides in triticale mesophyll protoplasts

Nocodazole (10 μM), chloroquine (100 μM) and sodium azide (5 mM) are commonly used inhibitors for endocytosis. Fluorimetric studies showed none of these compounds had significant inhibitory effect on translocation of either Tat or Tat_2 in mesophyll protoplasts (Fig. 5).

3.4. Competition experiments

Two sets of competition assays were performed using labeled and non-labeled peptides. In the first set, concentration of the labeled peptide was varied from 0 to 5 μM with respect to

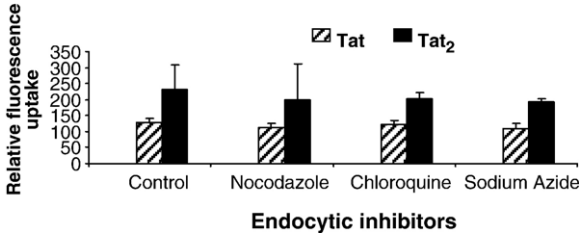

Fig. 5. Effect of endocytic inhibitors on permeation of Tat peptides in triticale mesophyll protoplasts; Nocodazole (10 μM), chloroquine (100 μM), sodium azide (5 mM).

the non-labeled peptide, keeping final concentration constant for the total peptide added (5 μM). The results revealed that nuclear accumulation of the fluorescence was dependent on the concentration of fluoresceinated peptide, irrespective of the concentration of non-labeled peptide added (Fig. 6). In the second set, the concentration of labeled peptide was maintained constant where as concentration of the non-labeled peptide was increased. Fluorescence microscopy and fluorimetric analysis showed no change in the nuclear accumulation of fluorescence (5 μM Tat or Tat_2) even in the presence of four times higher concentration of non-labeled peptide (Figs. 6D, Fig. 7A and B) further indicating receptor-independent, free, passive nuclear transport of the Tat peptides.

4. Discussion

Structural and functional studies for HIV-1 Tat basic domain (also a PTD) have been carried out extensively in mammalian cell lines, however, there are sporadic reports on cell-penetrating peptides in general and Tat in particular, in the plant system.

The present investigation shows efficient translocation and nuclear accumulation of fluoresceinated monomer and dimer of Tat basic domain in triticale mesophyll protoplasts. Cellular internalization of the dimer (Tat_2) was higher by 1.6 times than the Tat monomer. Studies have shown that presence of at least six arginine residues is required for effective translocation of Tat and with the increase in the number of arginine residues, the translocation ability also increases [9,34]. In the present study, it is possible that higher translocation ability of Tat_2 than Tat is due to higher number of arginine residues. Also, gene transfer efficiency of Tat dimer in the presence of standard transfection agent was found higher in human bronchoepithelial cells than the other oligomers [23]. Multivalency as in case of immunoglobulins may also enhance the functionality of cationic peptides by increasing cell penetrability and nuclear accumulation of cargo complexes [35].

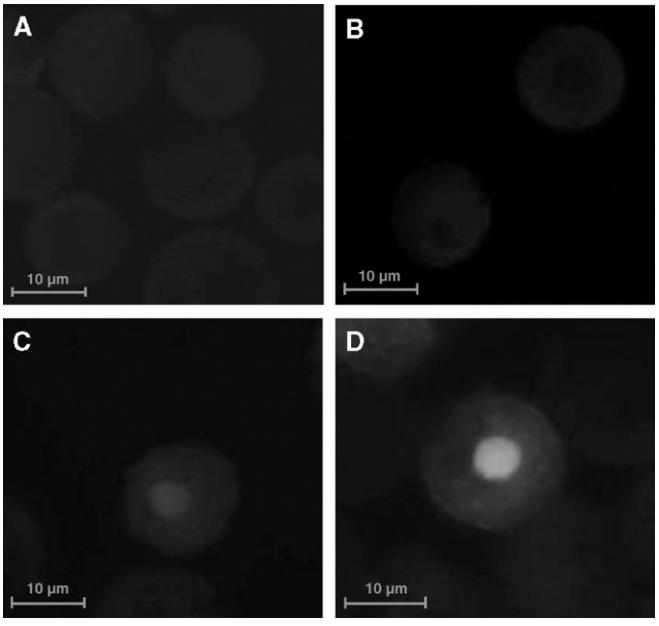

Fig. 6. Competition experiment with non-labeled Tat showing free nuclear transport of Tat peptide. Protoplasts were treated with fluorescently labeled and non-labeled Tat in different ratios (please see text for details). (A) Control; (B) non-labeled Tat; (C) total concentration of peptide 5 μM; 3 μM labeled Tat mixed with 2 μM of non-labeled Tat (3:2), note the weak fluorescence in nucleus is due to low concentration of the labeled Tat used. (D) 5 μM of labeled Tat mixed with 20 μM non-labeled peptide (1:4). Similar results were obtained with Tat_2.

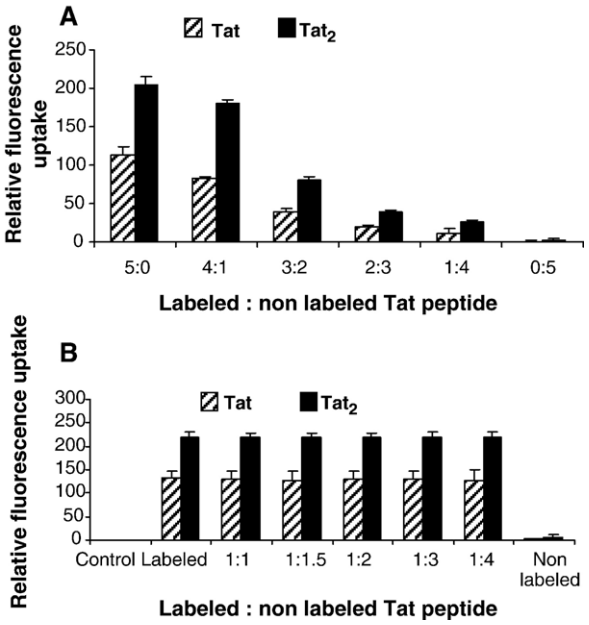

Fig. 7. Competition experiments suggested free, passive nuclear transport of Tat peptides. (A) Total peptide concentration was kept constant (5 μM) and the ratio of labeled and non-labeled peptide was varied. (B) concentration of the labeled peptide was fixed (5 μM) and concentration of the non-labeled peptide was increased maximum up to 20 μM.

The sequence specific effect was most noticeable in mutated Tat (M-Tat) which served as an excellent negative control. Substitution of first arginine residue with alanine (M-Tat) diminished both cell penetration as well as nuclear localization properties of Tat, further confirming that arginine residue play an important role in cell penetration ability of Tat peptide.

Tat and Tat_2 in the present study were internalized by the mesophyll protoplasts and showed nuclear accumulation within 10 min of incubation. Histones with much greater size than the cell-penetrating peptides have been reported to rapidly translocate and accumulate in the nucleus of petunia protoplasts [36].

Mechanism of translocation of Tat and cell-penetrating peptides across plasma membrane in animal cells still remains unclear [37–40]. Cell-penetrating peptides are speculated to enter the cell via non-endocytic pathways without any energy requirements and show increased accumulation in the cells with increase in their concentration. In the present study also, cellular internalization of Tat peptides was concentration-dependent and non-saturable. However, M-Tat showed no significant increase in cellular uptake even at the highest concentration, again demonstrating the significance of arginine residue in the cell penetration ability of the Tat basic domain.

It is speculated that cellular internalization of cationic peptides may require two steps: (1) electrostatic interaction with membrane phospholipids (2) translocation across the lipid bilayer by membrane destabilization [41]. Presence of extra positive charges may change physico-chemical properties of the membrane phospholipid bilayer resulting in enhanced permeability [42]. It is possible that excessive peptides enter the cell by a non-elucidated biological mechanism by interacting with other cell surface components [43]. Penetrating and conformational investigations reveal that translocation process of amphipathic cationic peptides may involve formation of β-barrel pore like structures or an association of helices in biomembranes [44].

Temperature also influenced the cellular internalization of Tat and Tat_2 in mesophyll protoplasts. Cellular uptake remarkably increased at 4 °C, as observed in mammalian cells. Histones have also been reported to show higher cellular uptake at low temperatures in petunia protoplasts [36]. Stimulated uptake may be due to phase transfer of biomembrane phospholipids and stabilization of inverted phospholipids micelles at low temperature [45,46]. We also studied the effect of peptide treatment on protoplast viability at various temperatures. Protoplasts like other plant explants are normally cultured between 20 °C and 28 °C as they are known to loose viability at higher temperatures such as 37 °C. In the present study also, the control as well the treated protoplasts maintained their viability in the temperature range 4 °C–24 °C suggesting that cellular uptake of peptides in protoplasts occurs via common non-endocytic mechanism at all the temperatures. The decrease in the fluorescence uptake at 37 °C was mainly due to loss of protoplast viability by incubation at high temperature than due to the peptide treatment. Arginine-rich intracellular delivery peptides containing fusion proteins also did not show any cytotoxic effect in onion and tomato cells [30].

In plants, endocytosis is now a well established basic cellular phenomenon [47–50]. Nocodazole, chloroquine, sodium azide inhibit the endocytic process in cells in different ways. Pre-incubation of triticale mesophyll protoplasts with these endocytic inhibitors did not result in any significant change in the cellular internalization of Tat and Tat_2 peptide. These studies along with stimulated cellular uptake of peptides at low temperature suggests that the process is receptor-independent and may not involve endocytic pathways.

Competition experiments between the labeled and non-labeled peptides ruled out the possibility of involvement of active nuclear transport of Tat peptides in mesophyll protoplasts. Increase in the concentration of non-labeled peptide did not cause inhibition of nuclear accumulation of labeled peptide indicating that the nuclear transport is receptor-independent and probably passive in nature.

Dual ability of cell-penetrating peptides such as Tat basic domain to translocate across the plasma membrane with macromolecular cargo complexes and also act as nuclear localization signal, has demonstrated their potential to revolutionize the field of bionanomedicine research [51–56]. In plants, emergence of reports in the last two years on cellular internalization of cell-penetrating peptides (penetratin, transportan and pVEC) and histones [29,36], fusion proteins with cell-penetrating peptides [30,57] and arginine rich oligopeptide mediated delivery of dsRNA [31] show that cell-penetrating peptides indeed could be the 'peptides of future' for studying various fundamental and biotechnological processes in plants. Cereals such as wheat and triticale are challenging targets for genetic manipulations through conventional methods of transformation. Cell-penetrating peptides such as Tat basic domain can be valuable vectors for alternate, simple and cost-effective strategies for gene transfer in these commercially important plant crops.

Also, similar to cell-penetrating peptides, virE2 protein of *Agrobacterium* (natural genetic engineer of plants) has nuclear localization sequence (bipartite) and can mediate nuclear uptake of ssDNA in plant cells. It will be interesting to carry out comparative study of the mechanisms of nuclear gene delivery in plants by virE2 and nuclear targeting cell-penetrating peptides.

In conclusion, this is the first report of characterization of cellular internalization of monomer and dimer of Tat basic domain in plant mesophyll protoplasts. Present investigations also show significance of sequence of Tat basic domain for cellular internalization in plants. Cell-penetrating peptides such as Tat can be used as nanocarriers for delivery of important macromolecules and hold tremendous potential for developing an entirely new field in plant sciences in the near future, that may be aptly called as 'phyto-nanobiotechnology'!

Acknowledgements

The technical assistance provided by Eric Amundsen is greatly appreciated. AC is thankful to Natural Science and Engineering Research Council of Canada (NSERC) for the award of Visiting Fellowship. AC is also thankful to Dr. Bob Parker (Alberta Peptide Institute) for his valuable suggestions regarding peptide synthesis. Dr. Fran Leggett (Lethbridge Research Centre) and Doug Bray (University of Lethbridge) provided timely help with epifluorescence microscopy. Financial support from MII program is gratefully acknowledged.

References

[1] A.I. Dayton, J.G. Sodroski, C.A. Rosen, W.C. Goh, W.A. Haseltine, The trans-activator gene of the human T cell lymphotropic virus type III is required for replication, Cell 44 (1986) 941–947.
[2] J. Hauber, A. Perkins, E.P. Heimer, B.R. Cullen, Transactivation of human deficiency virus gene expression is mediated by nuclear events, Proc. Natl. Acad. U. S. A. 84 (1987) 6364–6368.
[3] S. Ruben, A. Perkins, R. Purcell, K. Joung, R. Sia, R. Burghoff, W.A. Haseltine, C.A. Rosen, Structural and functional characterisation of human immunodeficiency virus *tat* protein, J. Virol. 63 (1989) 1–8.
[4] A.D. Frankel, C.O. Pabo, Cellular uptake of the Tat protein from human immunodeficiency virus, Cell 55 (1988) 1189–1193.
[5] M. Green, P.M. Lowenstein, Autonomous functional domains of chemically synthesised human immunodeficiency virus Tat trans-activator protein, Cell 55 (1988) 1179–1188.
[6] E. Vives, P. Brodin, B. Lebleu, A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus, J. Biol. Chem. 272 (1997) 16010–16017.

[7] K.T. Jeang, H. Xiao, E.A. Rich, Multifaceted activities of the HIV-1 transactivator of transcription, Tat, J. Biol. Chem. 27 (1999) 28837–28840.
[8] S. Schwarze, S. Dowdy, In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA, Trends Pharmacol. Sci. 21 (2000) 45–48.
[9] P.A. Wender, D.J. Mitchell, K. Pattabiraman, E.T. Pelkey, L. Steinman, J.B. Rothbard, The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters, Proc. Natl. Acad. U. S. A. 97 (2000) 13003–13008.
[10] E. Vives, Present and future of cell peptide mediated delivery systems: "Is Trojan horse too wild to go only to Troy?", J. Controlled Release 109 (2005) 77–85.
[11] C. De Coupade, A. Fittipaldi, V. Chagnas, M. Michel, S. Carlier, E. Tasciotti, A. Darmon, D. Ravel, J. Kerasey, M. Giacca, F. Cailler, Novel human derived cell-penetrating peptides for specific subcellular delivery of therapeutic biomolecules, Biochem. J. 390 (2005) 407–418.
[12] S. Fawell, J.M. Seery, Y. Daikh, C. Moore, L.L. Chen, B. Pepinsky, J. Barsoum, Tat-mediated delivery of heterologous proteins into cells, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 648–664.
[13] S.R. Schwarze, A. Ho, A. Vocero-Akbani, S.F. Dowdy, In vivo protein transduction: delivery of a biologically active protein into the mouse, Science 285 (1999) 1569–1572.
[14] M.A. Lindsay, Peptide-mediated cell delivery: application in protein target validation, Curr. Opin. Pharmacol. 2 (2002) 587–594.
[15] Y. Yang, J. Ma, Z. Song, M. Wu, HIV-1-mediated protein transduction and subcellular localization using novel expression vectors, FEBS Lett. 532 (2002) 36–44.
[16] B. Albarran, R. To, P.S. Stayton, A TAT-streptavidin fusion protein directs uptake of biotinylated cargo into mammalian cells, Protein Engg. Design Sel. 18 (2005) 147–152.
[17] A. Astriab-Fisher, D. Sergueev, M. Fisher, B.R. Shaw, R.L. Juliano, Conjugates of antisense oligonucleotides with the Tat and Antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences and biologic actions, Pharm. Res. 19 (2002) 744–754.
[18] V.P. Torchilin, T.S. Levchenko, R. Rammohan, N. Volodina, B. Papahadjopoulos-Sternberg, G.G.M. D'Souza, Cell transfection in vitro and in vivo with non-toxic TAT peptide–liposome–DNA complexes, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 1972–1977.
[19] J. Fominaya, M. Gasset, R. Garcia, F. Roncal, J.P. Albar, A. Bernad, An optimised amphiphilic cationic peptide as an efficient non-viral gene delivery vector, J. Gene. Med. 2 (2000) 455–464.
[20] C.H. Tung, S. Mueller, R. Weissleder, Novel branching membrane translocational peptide as gene delivery vector, Bioorg. Med. Chem. 10 (2002) 3609–3614.
[21] I. Hellgren, J. Gorman, C. Sylven, Factors controlling the efficiency of Tat-mediated plasmid DNA transfer, J. Drug Targeting 12 (2004) 39–47.
[22] I.A. Ignatovich, E.B. Dizhe, A.V. Pavlotskaya, B.N. Akiflev, S.V. Burov, S.V. Orlov, A.P. Perevozchikov, Complexes of plasmid DNA with basic domain 47–57of the HIV-1 Tat protein are transferred to mammalian cells by endocytosis-mediated pathways, J. Biol. Chem. 278 (2003) 42625–42636.
[23] C. Rudolph, C. Planks, J. Lausier, U. Schillinger, R.H. Muller, J. Rosenecker, Oligomers of the arginine-rich motif of the HIV-1TAT protein are capable of transferring plasmid DNA into cells, J. Biol. Chem. 278 (2003) 11411–11418.
[24] J.S. Wadia, S.F. Dowdy, Protein transduction technology, Curr. Opin. Biotechnol. 13 (2002) 52–56.
[25] D.A. Mann, A.D. Frankel, Endocytosis and targeting of exogenous HIV-1 Tat protein, EMBO J. 10 (1991) 1733–1739.
[26] M. Silhol, M. Tyagi, M. Giacca, B. Lebleu, E. Vives, Different mechanisms for cellular internalization of the HIV1-Tat-derived cell-penetrating peptide and recombinant proteins fused to Tat, Eur. J. Biochem. 269 (2002) 494–501.
[27] S. Console, C. Marty, C. Garcia-Echeverria, R. Schwendener, K. Ballmer-Hofer, Antennapaedia and HIV TAT "protein transduction domains" promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans, J. Biol. Chem. 278 (2003) 35109–35114.
[28] T.B. Potocky, A.K. Menon, S.H. Gellman, Cytoplasmic and nuclear delivery of a TAT-derived peptide and a β-peptide after endocytic uptake into HeLa cells, J. Biol. Chem. 278 (2003) 50188–50194.
[29] M. Mae, H. Myrberg, Y. Jiang, H. Paves, A. Valkna, U. Langel, Internalisation of cell-penetrating peptides into tobacco protoplasts, Biochim. Biophys. Acta 1669 (2005) 101–107.
[30] M. Chang, J.-C. Chou, H.-J. Lee, Cellular internalization of fluorescent proteins via Arginine-rich intracellular delivery peptide in plant cells, Plant Cell Physiol. 46 (2005) 482–488.
[31] N. Unnamalai, B.G. Kang, W.S. Lee, Cationic oligopeptide-mediated delivery of dsRNA for post-transcriptional gene silencing in plant cells, FEBS Lett. 566 (2004) 307–319.
[32] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 18 (1962) 100–127.
[33] R.S. Hodges, P.D. Semchuk, A.K. Taneja, C.M. Kay, J.M. Parker, C.T. Mant, Protein design using model synthetic peptides, Pept. Res. 1 (1988) 19–30.
[34] S. Futaki, Oligoarginine vectors for intracellular delivery: design and cellular-uptake mechanisms, Biopolymers 84 (2006) 241–249.
[35] M. Sung, G.M.K. Poon, J. Gariepy, The importance of valency in enhancing the import and cell routing potential of protein transduction domain-containing molecules, Biochim. Biophys. Acta 1758 (2006) 355–460.
[36] J. Rosenbluh, S.K. Singh, Y. Gafni, A. Graessmann, A. Loyter, Non-endocytic penetration of core histones into petunia protoplasts and cultured cells: a novel mechanism for the introduction of macromolecules into plant cells, Biochim. Biophys. Acta 1664 (2004) 230–240.
[37] U. Langel (Ed.), Cell-penetrating peptides: processes and applications, CRC press LLC, USA, 2002, p. 406.
[38] T. Suzuki, S. Futaki, M. Niwa, S. Tanaka, K. Ueda, Y. Sugiura, Possible existence of common internalization mechanisms among arginine-rich peptides, J. Biol. Chem. 277 (2002) 2437–2443.
[39] G. Drin, S. Cottin, E. Blanc, A.R. Rees, J. Temsamani, Studies on the internalization mechanism of cationic cell-penetrating peptides, J. Biol. Chem. 278 (2003) 31192–31201.
[40] E. Vives, J.P. Richard, C. Rispal, B. Lebleu, Tat peptide internalization: seeking mechanisms of entry, Curr. Protein Pept. Sci. 4 (2003) 125–132.
[41] G. Dom, C. Shaw Jackson, C. Matis, O. Bouffioux, J.J. Picard, A. Prochiantz, M.-P. Mingeot-Leclercq, R. Brasseur, R. Rezsohazy, Cellular uptake of Antennapedia penetratin peptides is a two-step process in which phase transfer precedes a tryptophan-dependent translocation, Nucleic Acids Res. 31 (2003) 556–561.
[42] S. Cohen, M.C. Bano, M. Chow, R. Langer, Lipid–alginate interactions render changes in phospholipid bilayer permeability, Biochim. Biophys. Acta 1063 (1991) 95–102.
[43] G. Drin, M. Mazel, P. Clair, D. Mathieu, M. Kaczorek, J. Temsamani, Physico-chemical requirements for cellular uptake of pAntp peptide: role of lipid-binding affinity, Eur. J. Biochem. 268 (2001) 1304–1314.
[44] S. Deshayes, M.C. Morris, G. Divita, F. Heitz, Interactions of amphipathic CPPs with model membranes, Biochim. Biophys. Acta 1758 (2006) 328–335.
[45] J.P. Berlose, O. Convert, D. Derossi, A. Brunissen, G. Chassaing, Conformational and associative behaviours of the third helix of antennapedia homeodomain in membrane-mimetic environments, FEBS J. 242 (1996) 372–386.
[46] C. Huang, S. Li, Calorimetric and molecular mechanics studies of the thermotropic phase behavior of membrane phospholipids, Biochim. Biophys. Acta 1422 (1999) 273–307.
[47] N.H. Battey, N.C. James, A.J. Greenland, C. Brownlee, Exocytosis and endocytosis, Plant Cell 11 (1999) 643–660.
[48] A. Bahaji, M.-J. Cornejo, E.O. Zapater, I. Contreras, F. Aniento, Uptake of endocytic markers by rice cells: variation related to growth phase, Eur. J. Cell Biol. 80 (2001) 178–186.
[49] J. Samaj, F. Baluska, B. Voigt, M. Schlicht, D. Volkmann, D. Menzel, Endocytosis. Actin cytoskeleton and signalling, Plant Physiol. 135 (2004) 1150–1161.
[50] J Samaj, F Baluska, D Menzel (Eds.), The plant endocytosis, Springer-Berlin, Heidelberg, 2005, p. 314.
[51] M.A. Zanta, P. Belguise-Valladier, J.P. Behr, Gene delivery: a single

nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 91–96.
[52] K.H. Bremmer, L.W. Seymour, C.W. Pouton, Harnessing nuclear localization pathways for transgene delivery, Curr. Opin. Mol. Therap. 3 (2001) 170–177.
[53] M. Nakanishi, A. Eguchi, T. Akuta, E. Nagoshi, S. Fujita, J. Okabe, T. Senda, M. Hasegawa, Basic peptides as functional components of non-viral gene transfer vehicles, Curr. Protein Pept. Sci. 4 (2003) 141–150.
[54] E. Hebert, Improvement of exogenous DNA nuclear importation by nuclear localization signal-bearing vectors: a promising way for non-viral gene therapy? Biol. Cell 95 (2003) 59–68.
[55] D.A. Dean, W.E. D.D.Strong, Nuclear entry of nonviral vectors, Gene Ther. 12 (2005) 881–890.
[56] A. Rolland, Nuclear gene delivery: the Trojan horse approach, Expert Opin. Drug. Deliv. 3 (2006) 1–10.
[57] T.-G. Kim, W.H.R. Langridge, Synthesis of an HIV-1 Tat transduction domain-rotavirus enterotoxin fusion protein in transgenic potato, Plant Cell. Rep. 22 (2004) 382–387.