International Journal of Biological Chemistry

ISSN 1819-155X

Academic Journals Inc.

www.academicjournals.com

International Journal of Biological Chemistry 1 (1): 47-53, 2007
ISSN 1819-155X

Improved *in vitro* Membrane Translocation Peptide Assays

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Abstract: In this study, we had tried various methods, including acidic solution wash, trypsin digestion and typan blue fluorescence quenching, to eliminate false results in cell membrane translocation assays *in vitro*. Although both trypsin digestion and typan blue fluorescence quenching methods had certain effects, none of them was able to totally eliminate the artifact in FACS and confocal microscopy assays. On the contrary, acidic solution wash could remove cell membrane bound peptide efficiently and proved to be a reliable procedure for membrane translocation peptide assays *in vitro* using FACS and confocal microscopy.

Key words: Membrane penetration, peptide, translocation, fluorescence microscopy, fluorescence-activated cell sorting, delivery, conjugation

INTRODUCTION

Cell translocation (penetrating) peptides are a family of recently identified proteins/peptides. These peptides are able to translocate across the plasma membrane and get into live cells without affecting cellular integrity. Since cell translocation peptide permits cargo of conjugated peptides, oligonucleotides and even proteins into cell without affecting the cell integrity (Lundberg and Johansson, 2001; 2002), it has become an invaluable tool in applied cellular biology, including drug delivery (Liang and Yanga, 2005a,b; Park *et al*., 2005).

Cell translocation proteins/peptides were originally isolated from infective organisms such as bacteria or viruses (e,g., HIV TAT protein and the HBV VP22 protein) with a common feature of being rich in positively charged amino acid residues (Liang and Yang, 2005a, b; Park *et al*., 2005). With this knowledge, highly positive charged peptides rich in Arg or Lys have been synthesized and have also been reported to possess cell penetrating activity (Matsui *et al*., 2003; Mi, 2000). As more and more peptides are claimed to possess potential cell penetrating ability, data from *in vitro* and *in vivo* studies and from different experiments of the same peptide have yielded controversial and confusing results (Lundberg and Johansson, 2001; Shenk, 2002; Elliott and O'Hare, 1997; Schwarze *et al*., 1999; Normand *et al*., 2001; Aints *et al*., 1999; Phelan *et al*., 1998). It has been shown that although some of these peptides or fused proteins show very strong *in vitro* cell penetrating activity, they hardly showed any cell translocation activity *in vivo* (Elliott and O'Hare, 1997; Schwarze *et al*., 1999; Normand *et al*., 2001; Aints *et al*., 1999). Recent studies suggested that cell fixation procedures in fluorescence microscopy and fluorescence-activated cell sorting (FACS) assays could induce an artifact membrane translocation for these membrane-bound peptides due to their positively charged nature. Transportation of membrane-bound peptide into cytoplasm caused false results in membrane penetrating peptide assays *in vitro* (Lundberg and Johansson, 2001, 2002; Richard *et al*., 2003). In this study, methods to reduce or eliminate artifacts associated *in vitro* membrane translocation peptide assays had been tried and compared and reliable method was presented.

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MATERIALS AND METHODS

Materials

HeLa and MOLT-4 cell lines were from American Type Cell Collection (ATCC). Alexa Fluor-488 fluorescent-labeled histone H1 (His-FITC) and 5-(bromomethyl) fluorescein was from Molecular Probes Inc (Eugene, OR, USA).

Cell Culture

HeLa cells were cultured in Minimum essential medium (Eagle) with 2 mM L-glutamine, Earle's BSS and 10% fetal bovine serum.

Peptide Synthesis and Fluorescent Labeling

Peptide TAT, the Protein Transduction Domain (PTD) of the human immunodeficiency virus (HIV) protein TAT, was synthesized by solid-phase peptide synthesis using AEDI-Expansin resin with a 9050 Pep Synthesizer (Millipore, Wartford, UK) according to the Fmoc/tBoc method and was purified using reverse phase HPLC. In addition to the core sequence, a cysteine residue was introduced at the N-terminal of TAT [CYGGRRKKRRR] for fluorescent labeling. Such TAT peptide was fluorescent labeled through the thiol group of cysteine by reaction with 5-(bromomethyl) fluorescein (molar ratio 1:4) at 4°C for overnight. Fluorescence labeled TAT was purified on FPLC equipped with a heparin column through a gradient elution with 2.0 M NaCl (Liang and Yang, 2005b). Samples were desalted through ultra-filtration (MW cut = 1,000) and then freeze-dried.

Fluorescence Microscopy

HeLa cells were plated on a slide chamber 24 h before each experiment. Cells were exposed to various peptides (5 μM) at 37°C for 30 min. After removal of the culture medium, cells were washed with various solutions (acid or base), followed by Hank's solution (pH = 7.4). Fixation was done with 100% methanol at 4°C for 15 min. Examination was done with an inverted fluorescence microscope (Zeiss, Gottingen, Germany) as descried previously (Liang and Yang, 2005b).

Fluorescence-activated Cell Sorting (FACS)

Cells were incubated with various peptides (5 μM) at 37°C for 30 min. After removal of culture medium by centrifugation, cell pellets were suspended in various wash solutions (acid or base) and then collected again by centrifugation. Cells were suspended in Hank's solution (pH = 7.4) for FACS assay. Cells were analyzed with a BD Biosciences FACS Calibur system (BD Biosciences, Mountain View, CA) as described previously (Park *et al*., 2005). FACS experiments were done in The University of Michigan at Ann Arbor, USA.

RESULTS AND DISCUSSION

Since the transportation of membrane-bound peptide into cytoplasm during the cell fixation step has been suggested as the reason for the false result in membrane penetrating peptide assays *in vitro* (Lundberg and Johansson, 2001; 2002; Richard *et al*., 2003), we focused on methods to remove cell membrane bound peptides or to discriminate them from peptides translocated inside of cells. Histone H1, a positively charged peptide having strong membrane binding ability but without membrane-penetration ability (Lundberg and Johansson, 2001; 2002), was selected as a negative control. To quantify the binding of fluorescent labeled histone H1 (His-FITC) to cell membranes, an event window (M1) was set in the FACS histogram to distinguish His-FITC bound cells from unstained cells (Fig. 1A). More than 96% of His-FITC bound cells but less than 5% of the unstained cells fell in this window. The removal of cell membrane bound His-FITC could be reflected by decreased event numbers in this M1 window.

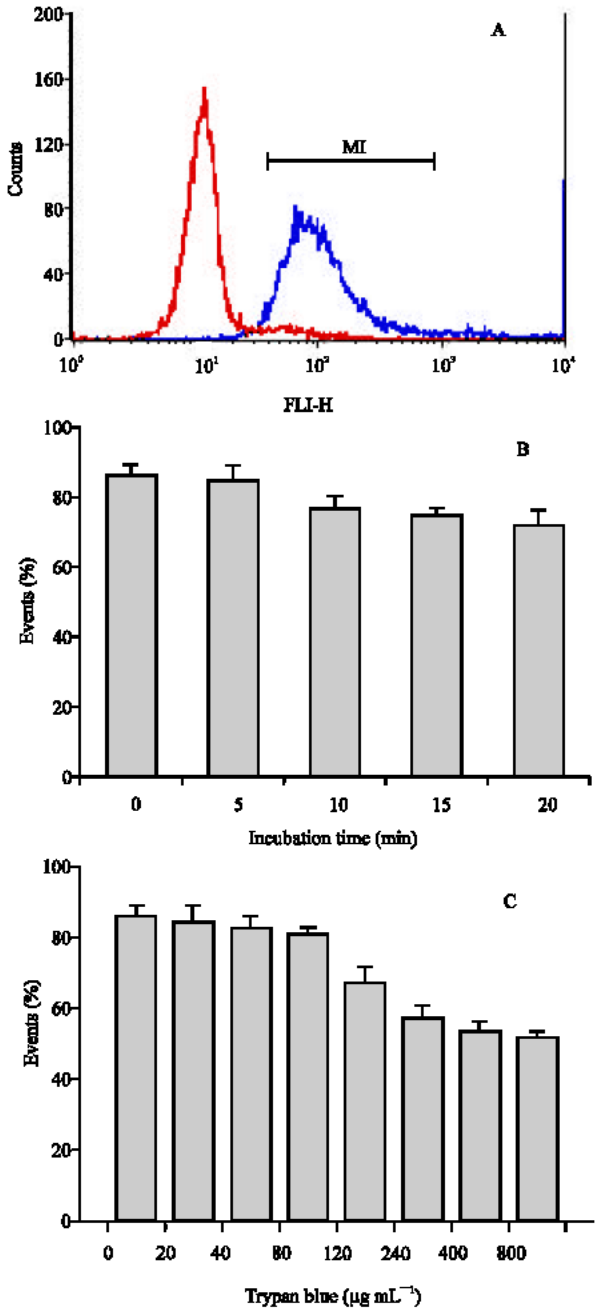

Fig. 1: FACS assay of His-FITC treated cells. Cells were exposed to 5 μM of His-FITC at 37°C for 30 min and were then subjected to FACS analysis. A) a event window (M1) was set in FACS histogram to represent His-FITC bound cells (Blue line), unstained cells (Red line); B) after incubation with His-FITC, His-FITC treated cells were incubated with 0.25% trypsin at 37°C for up to 20 min. At various time intervals, cells were collected, washed and then subjected to FACS assay; C) after incubation with His-FITC, His-FITC treated cells were incubated with trypan blue at various concentrations for 5 min before FACS assay. Event % = events in set window (M1)/total events ×100

Trypsin digestion of membrane-bound peptide was first examined. Cell suspensions were incubated with His-FITC at 37°C for 30 min. After that, cells were treated with 0.25% trypsin for up to 20 min. At various time intervals, cells were collected, washed and then subjected to FACS assay. As shown in Fig. 1B, trypsin treatment caused a fluorescence intensity drop (i.e., event percentage drop in M1 window) in His-FITC treated cells, implying the degradation of cell surface bound His-FITC. Increasing trypsin digestion time had a positive but limited effect. Only about 20% of cell membrane bound His-FITC was removed even when cells were incubated with trypsin for up to 20 min. Neither higher trypsin concentration nor longer incubation time was examined in this study with the consideration that over-digestion may cause serious cell membrane damage and affect entire cell biological and mechanical functions. Quenching of cell membrane bound His-FITC by trypan blue was also attempted. Since trypan blue can not enter into live cells and only cell membrane surface associated fluorescence can be quenched, trypan blue does have the potential to discriminate peptides inside of cells from those bound to the cell membranes. Cells were treated with His-FITC for 30 min and then incubated with various concentrations of trypan blue for 5 min before they were subjected to FACS assay. As shown in Fig. 1C, although greater surface associated fluorescence of His-FITC could be quenched with higher concentration of trypan blue, only less than half of the His-FITC fluorescence could be quenched even when as high as 0.8 mg mL^{-1} of trypan blue was used.

As we know, the interaction between membrane bound peptides and cell membranes can not be limited to cell membrane surfaces. Peptides on cell surfaces may partially insert into the lipid bi-layer of cell membranes. In such a case, membrane bound peptides can hardly be are accessed and cleaved by trypsin (Craig *et al*., 1994). Although small molecule such as trypan blue has a greater chance of reaching cell membrane-bound peptides compared to big enzyme molecule, it may still be unable to access fluorescent probes which are inserted in the membrane or buried in the hydrophobic areas of cell membranes. Therefore, even though both trypsin and trypan blue methods could be used; their efficiency in reducing artifacts in membrane translocation peptide assays *in vitro* should be limited.

Removal of membrane-bound peptide was also tried using either an acidic or a base solution based on the hypothesis that changing the electrostatic status of peptides or cell membranes may disturb their interactions. In these experiments, after incubation with His-FITC, cells were washed three times with either an acidic or a base solution on ice. An additional washing step with Hank's solution (pH = 7.4) prior to FACS assay was necessary to restore cell membrane pH. We found that an acid wash using a pH = 2.5 solution (28 mM NaAC, 120 mM NaCl, 20 mM barbital, pH = 2.5) exhibited high efficiency in removing cell membrane associated His-FITC. Most membrane-bound His-FITC were removed by acid wash, as justified from the comparable fluorescence intensity between His-FITC treated and untreated cells (Fig. 2A). The same acid wash approach was further tested on a well-demonstrated membrane translocation peptide, TAT. It was interesting to find that unlike in His-FITC treated cells, the acid wash only caused a slight fluorescence intensity drop in TAT-FITC treated cells (Fig. 2B). A reasonable explanation for this observation should be that most TAT-FITC entered into cells and thus could not be affected by the acid wash. Observed slight fluorescence intensity drop might reflect the removal of a small portion of membrane-bound TAT-FITC. Acid wash under described conditions would not cause significant cell viability change as demonstrated by followed trypan blue staining and MTT assays (data not shown).

The artifact elimination effect of acid wash on *in vitro* membrane translocation peptide assay was also tested using fluorescence microscopy. As shown in Fig. 3, both His-FITC and TAT-FITC exhibited cell membrane translocation ability using the regular methanol fixation procedure without acid wash, as justified from the bright fluorescence inside His-FITC (Fig. 3A) and TAT-FITC (Fig. 3B) treated cells. This result was in accordance with previous findings (Lundberg and Johansson, 2001; 2002; Richard *et al*., 2003) and confirmed the artifacts in membrane translocation peptide assay *in vitro*. However if the acid washing step was introduced in the slide preparation before cell fixation,

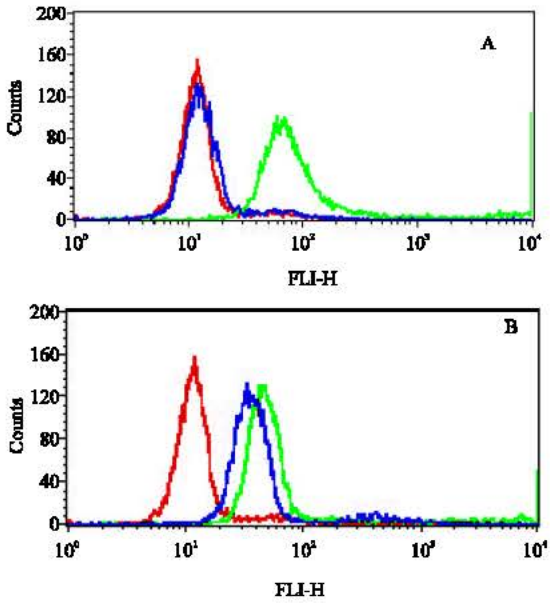

Fig. 2: FACS assay of His-FITC (A) and TAT-FITC (B) treated cells washed with different solutions. Green line, cells treated with 5 μM His-FITC (A) or TAT-FITC at 37°C for 30 min and then washed with Hank's solution (pH = 7.4); Blue line, cells were treated with 5 μM His-FITC (A) or TAT-FITC at 37°C for 30 min and then washed with acidic solution (28 mM NaAC, 120 mM NaCl, 20 mM barbital, pH = 2.5) for three times, followed by Hank's solution (pH = 7.4); Red line, untreated cells

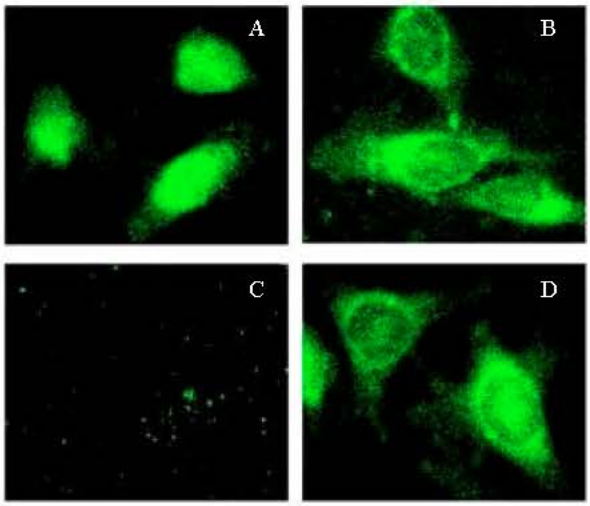

Fig. 3: Observation of His-FITC or TAT-FITC treated cells under fluorescence microscopy. Cells were treated with 5 μM His-FITC (A, C) or TAT-FITC (B, D) at 37°C for 30 min. Cells were washed with Hank's solution (A, B) or acidic solution (28 mM NaAC, 120 mM NaCl, 20 mM barbital, pH = 2.5) (C, D) before they were fixed using 100% methanol at 4°C for 20 min. Intracellular distributions of His-FITC and TAT-FITC were observed under fluorescence microscopy

no fluorescence could be observed inside His-FITC treated cells (Fig. 3C) while the fluorescence inside TAT-FITC treated cells were hardly affected (Fig. 3D). This result is in agreement with above FACS assay (Fig. 2) and confirms that acid washing procedure is an effective mean to reduce or avoid artifacts associated with *in vitro* membrane translocation peptide assays.

CONCLUSIONS

Artifacts in the *in vitro* membrane translocation peptide assays prove to be caused by fixation-induced translocation of membrane bound peptides. Although artifact elimination methods such as enzyme digestion and fluorescence quenching have been used by some investigators, none of them is able to totally eliminate the artifact in FACS and confocal microscopy assays. Cell treatment with an acidic solution to remove membrane bound peptides has proven to be an effective method which can completely avoid the artifacts in membrane translocation peptide assays using FACS and confocal microscopy.

ACKNOWLEDGMENT

This research was partially supported by an Upjohn Research Award to JFL.

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