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Evidence that membrane transduction of oligoarginine does not require vesicle formation

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Abstract

The involvement of vesicular formation processes in the membrane transduction and nuclear transport of oligoarginine is currently a subject of controversy. In this report, a novel quantitative method which allows for the selective measurement of membrane transduction excluding concurrent endocytosis was used to determine the effects of temperature, endosomal acidification, endosomolysis, and several known inhibitors of endocytic pathways on the internalization of oligoarginine. The results show that, unlike endocytosis, transduction of oligoarginine was not affected by incubation at 16°C as compared to the 37°C control, and was only partially inhibited at 4°C incubation. Additionally, membrane transduction was not inhibited to the same extent as endocytosis following treatment with ammonium chloride, hypertonic medium, amiloride, or filipin. The endosomolytic activity of oligoarginine was investigated by examining the leakage of FITC-dextran into the cytosolic compartment, which was not higher in the presence of oligoarginine. Furthermore, ammonium chloride showed no effect on the nuclear transport of oligoarginine. The data presented in this report indicate that membrane transduction is likely to occur at the plasma membrane without the formation of membrane vesicles, and the nuclear localization involves membrane transduction, rather than endocytosis of oligoarginine.

Keywords: Membrane transduction; Endocytosis; Oligoarginine; Membrane transduction peptides; Cell-penetrating peptides; Quantitative measurement

Introduction

Recently, peptides characterized as membrane transduction peptides (MTPs), or cell-penetrating peptides (CPPs), are of interest due to their ability to translocate across cellular membranes directly into the cytoplasm of cells in a novel mechanism termed membrane transduction. This membrane transduction process is of tremendous importance in the delivery of macromolecular drugs, including peptides, oligonucleotides, and genes, with cytoplasmic and nuclear targets [1–3]. Molecules capable of membrane transduction are short sequences of amino acids, usually from 9–30 amino acids, containing an abundance of arginine and lysine residues (recently reviewed in [4]). However, whether these MTPs are able to gain access to the cytoplasmic compartment directly via the plasma membrane, or across intracellular vesicles following internalization via endocytosis is still a subject of controversy [5,6]. Early studies of MTPs such as HIV-1 Tat-(47–57) [7], Antennapedia (43–58) [8], and small oligoarginine peptides [9,10], across cell membranes showed that the mechanism was temperature, energy, and

Abbreviations: α-MEM, α-minimum essential medium; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; CHO, Chinese hamster ovary; CPP, cell-penetrating peptide; E-64, (2*S*,3*S*)-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl)-oxirane-2-carboxylic acid; EDTA, ethylene-di-amine-tetra-acetic acid; E-64, trans-(epoxy-succinyl)-L-leucylamino-(4-guanidino)butane; FITC, fluorescein 5′-isothiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIV-1, human immunodeficiency virus type 1; MTP, membrane transduction peptide; NH_4Cl, ammonium chloride; PBS, phosphate-buffered saline; PMSF, phenyl-methylsulfonyl fluoride; RFU, relative fluorescent units.

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cell-type independent. These findings suggested an exclusion of endocytosis as a mechanism of membrane transduction. On the other hand, recent studies have implicated the involvement of several different types of endocytosis, including macropinocytosis, in the internalization of MTPs with varying results [5,11–15]. Most recently, it has been proposed that the guanidinium headgroups of arginine-containing peptides form bidentate hydrogen bonds on the cell surface, resulting in the migration across the lipid bilayer into the cytosol [16]. However, this hypothesis does not preclude endosomes as the location that the transmembrane transport occurs.

The discrepancy regarding the mechanism of membrane transduction is mostly due to problems with the experimental methods being used to measure the peptide internalization. The high plasma membrane surface binding and artifactual re-distribution following fixation have made many of the conclusions from previous studies questionable [17–19]. Moreover, the in vitro methods previously used may not quantitatively measure the membrane transduction efficiency of these MTPs since it is likely that membrane transduction occurs concurrently with endocytosis [16,20]. Many studies on the MTPs were based on the measurement of cytoplasmic activity of cargo macromolecules in the cell. However, the biological activity of cargo macromolecules may not accurately measure the membrane transduction efficiency, because the attachment of large macromolecules with varying chemical properties alters the internalization properties [21–24], possibly changing transduction and endocytosis to different extents. Consequently, there is no established quantitative correlation between the expression of biological activity of the cargo macromolecules and the transduction efficiency of the MTPs.

In order to obtain unambiguous results to determine the involvement of these two transport processes, we have recently developed a quantitative method to measure the membrane transduction efficiency, separately from endocytosis. The technique separates the vesicular versus cytoplasmic compartments, which allows for the calculation of the membrane transduction and the characterization of the structural requirements for several MTPs. Using this method, we have previously shown that while oligoarginine is primarily transduced into CHO cells, oligolysine is internalized predominantly by endocytosis [20]. This report describes the differential effect of temperature, pH, and several other inhibitors on internalization via endocytosis or membrane transduction of oligoarginine and oligolysine, and the subsequent nuclear localization of these oligopeptides.

Materials and methods

Peptide synthesis and labeling

$YG(R)_9$, and $YG(K)_9$ were synthesized by Genemed (South San Francisco, CA). The tyrosine moiety was labeled with Na-^{125}I (ICN, Irvine, CA) using the Chloramine-T method [25]. The labeled oligopeptides were purified by size exclusion chromatography using Sephadex G-15 (Sigma, St. Louis, MO) gel matrix.

Cell culture

Chinese hamster ovary (CHO) cells (ATCC, Manassas, VA) were grown in α-minimum essential medium (α-MEM) (GIBCO-BRL, Carlsbad, CA) containing 10% fetal bovine serum (GIBCO-BRL). The cells were replenished with fresh medium the day before confluence at which time the localization assays were performed.

Cytoplasmic localization assays

The measurement of cytoplasmic localization of MTPs was performed with a previously reported procedure [20]. Briefly, confluent CHO monolayers grown in T75 flasks (Corning, Acton, MA) were incubated in serum-free medium containing 5 μg/mL of ^{125}I-labeled oligopeptide, 0.1 mg/mL FITC-Dextran (70 kDa) (FD) (Sigma), and protease inhibitor cocktail (PI) (Sigma) containing 4 μM AEBSF, 2 μM EDTA, 0.3 μM bestatin, 30 nM E-64, 2 μM leupeptin, and 0.6 nM aprotinin. After treatment for 1 h at either 4, 16, or 37°C, the monolayers were washed three times with cold PBS, detached by treatment with trypsin-EDTA at 37°C for 5 min, and the isolated cell pellets washed with 0.5 mg/mL heparin-PBS followed by PBS. The cell pellets were then homogenized in buffer (HB) containing 0.25 M sucrose, 2 mM EDTA, and 10 mM HEPES, pH 7.4 using the Balch cell press (H & Y Enterprises, Redwood City, CA) [26]. The cell homogenate was centrifuged at 600 × *g* at 4°C for 10 min, and the post-nuclear supernatant was fractionated using Sephacryl S-500 (Amersham, Piscataway, NJ) size exclusion chromatography (1 × 13 cm column dimensions) with HB as the eluting buffer. 1-mL fractions were collected and assayed for ^{125}I-oligopeptides using a Gamma counter (Packard, Downers Grove, IL), for FD using fluorescence spectroscopy (Hitachi, Tokyo, Japan) (Ex 494 nm Em 519 nm), and for protein content using the Pierce protein assay kit. The amount of oligopeptide internalized by endocytosis versus transduction was calculated using the equations previously described [20]. For the endocytosis inhibitor assays, the localization assay method was performed after pre-treating CHO cell monolayers at 37°C with serum-free medium containing either 0.3 M sucrose for 15 min, or 50 mM NH_4Cl, 1.5 μM filipin, or 100 μM amiloride for 30 min, followed by a 1-h co-incubation with ^{125}I-oligopeptide and FD and the respective inhibitors. The internalization of ^{125}I-transferrin (Sigma) (3 μg/mL) and BODIPY-lactosylceramide (Molecular Probes) (2 μM) was measured following the above pre-treatments with sucrose and filipin as positive controls for clathrin- and caveolar-mediated endocytosis, respec-