Biochemical and Biophysical Research Communications 443 (2014) 1136–1140

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cellular uptake of the *Antennapedia* homeodomain polypeptide by macropinocytosis

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ARTICLE INFO

Article history:
Received 25 November 2013
Available online 18 December 2013

Keywords:
Antennapedia homeodomain
Penetratin
Cell-Penetrating Peptides (CPPs)

ABSTRACT

Antennapedia homeodomain has been shown to be able to translocate from extracellular space into the cytoplasm of cells in a receptor-independent manner. Its third α-helix domain, designated as "Penetratin", was proposed to be the functional transduction domain that is responsible for the translocation, and it is widely used for intracellular delivery of various exogenous proteins. Although Penetratin has been regarded to be the only element conferring the capacity on its parent polypeptide to penetrate through the plasma membrane, we found that the complete *Antennapedia* homeodomain exhibits an appreciably higher level of translocation efficiency as compared to Penetratin. Pharmacological analysis demonstrated that macropinocytic endocytosis plays a significant role underlying the process of the homeodomain internalization, and this is consistent with the observation that internalized polypeptide co-localizes with a fluid phase dye. Our results identify macropinocytosis as a major mechanism by which *Antennapedia* homeodomain obtains the access to the interior of cells, providing a novel perspective in the field of protein translocation and transduction.

1. Introduction

One of the most fundamental features of living organisms is that cells are separated from their external environment by a thin, but highly complex plasma membrane constituted of a lipid bilayer. Although, the lipid bilayer is only a few nanometers in width (5–10 nm), it is impermeable to most molecules apart from small hydrophobic ones. The ability of small molecules to diffuse through a lipid bilayer is related to their lipid solubility. Surprisingly, some recombinant proteins and peptides generated from *Escherichia coli*, such as the trans-activator of transcription (Tat) protein derived from human immunodeficiency virus-1 (HIV-1) [1] and the *Antennapedia* homeodomain (HD*Antp*) from *Drosophila* [2], can gain free access to the cytoplasm of mammalian cells, in a receptor-independent manner [3]. This phenomenon has attracted a lot of attention and it has been intensively investigated since its discovery more than two decades ago.

While the underlying cellular internalization mechanism remains controversial, the most widely accepted hypothesis proposed the existence of a specific transduction domain (TD) within each of these naturally occurring proteins that is responsible for transporting its parent cargo protein across the plasma membrane. For instance, the third α-helix of the HD*Antp* corresponding to residues 43–58 was suggested to be a TD by screening effective peptide fragments retaining the translocation ability [4], and has been designated as Penetratin. Collectively, the term Cell-Penetrating Peptides (CPPs) has been coined to refer to those TDs and to the other designed short peptides capable of penetrating into cells [5–7]. CPPs share some common features; they are short peptides of less than 20 amino acids, highly positively charged and have the ability of intracellular delivery of various cargos when conjugated to them or even in the unconjugated mode by forming a complex with the cargo molecule.

However, recent reports show that engineered supercharged GFP and other naturally supercharged human proteins with a ratio of charge units per kDa greater than 0.75 could deliver mCherry and other functional proteins with significantly higher efficiency than the conventional CPPs including Penetratin, oligoarginine, and TAT (TD derived from Tat) both in *vitro* and *in vivo* [8,9]. Since HD*Antp* has a remarkably high ratio of positive charges per kDa i.e. 1.54, this raises the question of whether the translocation capacity of Penetratin could truly represent that of its parent HD*Antp* polypeptide. Here, we compared the translocation efficiency of *Antennapedia* homeodomain with that of Penetratin, and assessed the underlying molecular mechanism pharmacologically, to gain a deeper insight into this intriguing macromolecular translocation phenomenon.

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0006-291X
http://dx.doi.org/10.1016/j.bbrc.2013.12.062

2. Materials and methods

2.1. Reagents

Methyl-β-cyclodextrin, 5-(*N*-Ethyl-*N*-isopropyl) amiloride, poly(deoxyinosinic-deoxycytidylic) acid sodium salt, genistein, dichloro diphenyl trichloroethane, Chlopromazine, imidazole, heparin were purchased from Sigma. [alpha-32P] Deoxyadenosine 5′-triphosphate (dATP) isotope was obtained from Hartman-analytic. Dextran Alex-647 was purchased from Invitrogen.

2.2. Cell lines and cell culture

Hela, Hela H2B::GFP, NMuMG, HEK 293T cell lines were cultured in Dulbecco's Modified Eagle's medium DMEM with high W/Glutamax-I (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), at 37 °C with 5% CO_2. Schneider 2 (S2) cells were cultured in Schneider's *Drosophila* Medium (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) at 25 °C without supplying CO_2.

2.3. Live cell imaging

Hela were seeded onto 8-well μ-Slide (ibidi) in 220 μl medium (1×10^4 per well). After 22 h, cells were washed once with PBS and incubated in serum-free DMEM for 30 min, or pretreated with the respective drugs, followed by incubation in protein solution for another 60 min. After incubation, cells were washed three times with 20 U/ml heparin in PBS to remove membrane-bound protein, and imaged in prewarmed growth medium. Cells were imaged on a Leica SP5 MP confocal microscope on a heated stage with either a 63 × or 100 × objective lens. Images were processed using ImageJ software.

2.4. Flow cytometry

Hela cells were plated onto a 24-well plate at a density of 6×10^4 cells per well. After 22 h, cells were incubated as described above for live imaging, then trypsinized, resuspended in 200 μl of growth medium and placed on ice. Cells were analyzed on an LSRII flow cytometer (BD Biosciences) for mCherry internalization (ex, 561 nm). Cells were gated for live cells and at least 10,000 live cells were analyzed for each treatment. The data were analyzed with FlowJo software (Tree Star, Inc.).

2.5. Electrophoretic mobility shift assay

According to the sequence of the D4 enhancer derived from *Drosophila*, oligonucleotides were designed as forward primer: 5′-agtttaccattaaattcccatttaggctgtcaatcatttgcgct-3′ and reversed primer as: 5′-aagccgccaagaaaaattagcgcaaatgattgacagcctaaatggg-3′ for Electrophoretic Mobility Shift Assay (EMSA). Oligonucleotides were synthesized by Microsynth (Switzerland) and then labeled with [α-32P] dATP (Hartman) using the Klenow fragment of DNA pol I (New England Biolabs). 10 ng of annealed oligonucleotide was used per reaction. Unincorporated label was removed with illustra MicroSpin G-25 Columns (GE). Poly (dI.dC) (Sigma) was used to reduce nonspecific binding. The resulting solution was analyzed by non-denaturing electrophoresis using a 6% acrylamide gel, and visualized with X-ray film.

3. Results and discussion

3.1. Characteristics of recombinant proteins

The primary sequence of HD*Antp* was used to calculate the ratio of net theoretical charge (lysine and arginine: +1; aspartate and

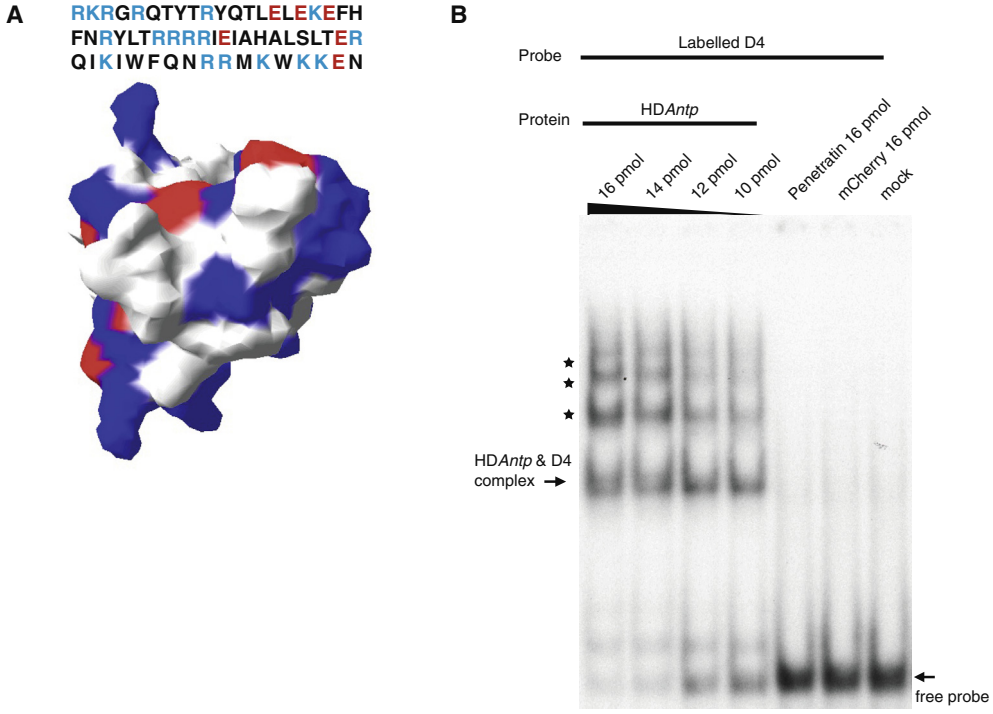

Fig. 1. Naturally supercharged *Antennapdia* homeodomain. (A) Surface charging of HD*Antp*. Blue indicates positively charged residues, whereas red indicates for negatively charged ones. The visualization of surface information is based on the entry of 9ANT from the Protein Data Base (PDB) using the program Protein Model Portal (PMP). (B) HD*Antp* specifically binding to D4 enhancer probe. From left to right, the complexes of HD*Antp* and D4 probe are decreased proportionally according to the loading amount of protein. The oligomerization of mCherry tag can be observed, as indicated by astride.

glutamate: −1) per kDa, yielding a value of 1.54 (Fig. 1A) [9]. In order to be able to compare the penetration efficiency of HD*Antp* and its transduction domain "Penetratin", we fused them to mCherry fluorescent proteins using the same linker and orientation (Sup. 1A) [8,10]. The cysteine39 in wild type HD*Antp* was replaced by serine to avoid the formation of artificial intermolecular dimers by disulfide bridges [11]. These fusion proteins retained their ability of rapid intracellular translocation from the medium without cytotoxicity (Sup. 1B and C). To ascertain whether the recombinant proteins maintained their specific DNA binding ability, [α-32P]dATP labeled probe D4 was used for Electrophoretic Mobility Shift Assay (EMSA). D4 consists of 62 base pairs of DNA corresponding to a fragment within the enhancer of *Spineless* (*Ss*) in *Drosophila* [12], which is bound by endogenous *Antp* and other co-transcription factors during leg development. As shown in Fig. 1B, recombinant HD*Antp* protein specifically binds to the 62-bp D4 probe, forming protein/DNA complex proportionally to the amount of protein added, whereas cationic Penetratin and mCherry showed no affinity for the negatively charged DNA oligos, indicating that recombinant HD*Antp* maintains the specific binding ability with the consensus DNA sequence.

3.2. HDAntp delivers mCherry with greater efficiency than Penetratin into cells

As fixation of cells causes artificial redistribution of CPPs [13], we monitored the cellular uptake always in live cells. Each recombinant protein was added to cell culture medium followed by 60 min of incubation. After washing with heparin containing PBS to remove proteins sticking to the cell surface [8], protein internalization was visualized by confocal fluorescence microscopy. HD*Anp* fusion protein showed stronger intracellular signals than that of Penetratin (Fig. 2A), whereas mCherry alone barely gave any detectable signal under the same microscope settings. To further quantify translocation efficiency of each protein, we applied fluorescence-assisted cell sorting (FACS) to the Hela cells under the

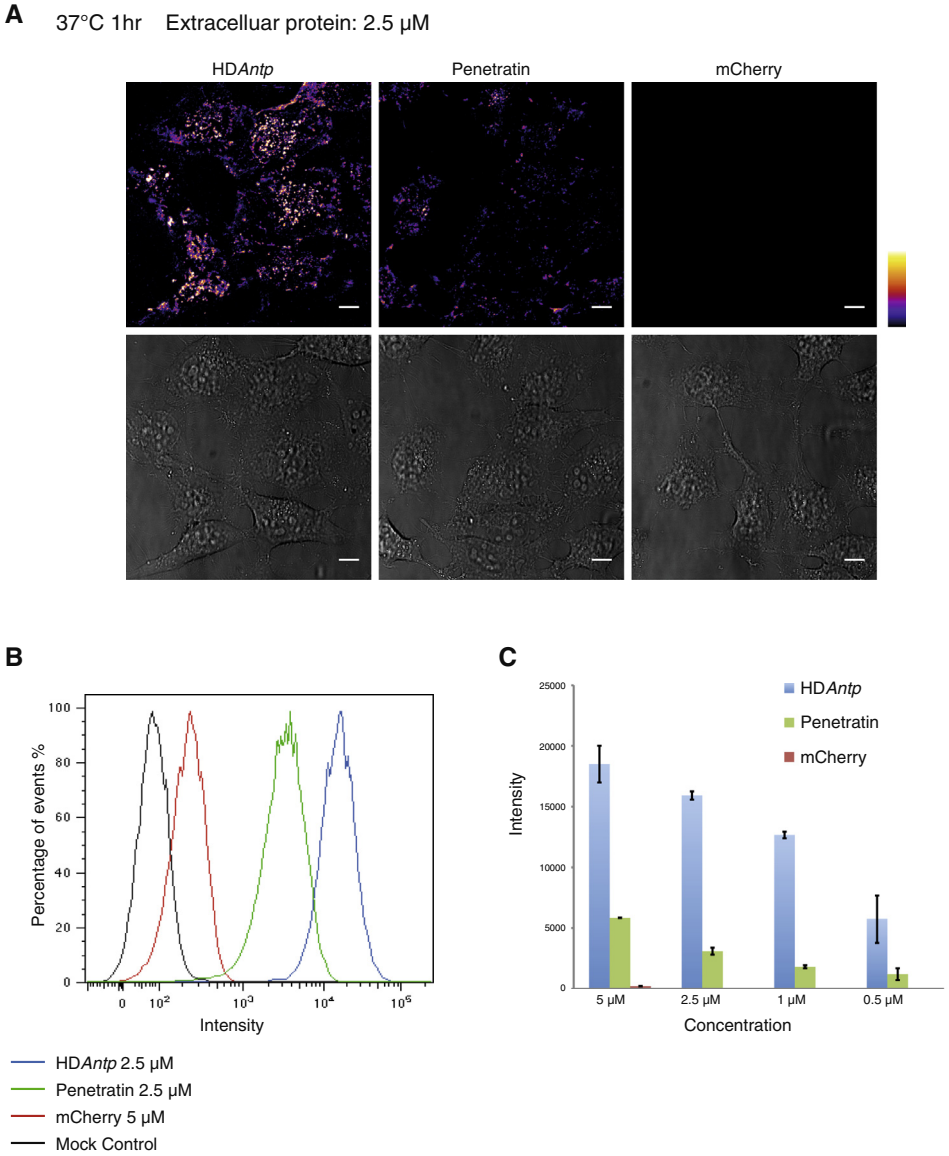

Fig. 2. HD*Antp* compared with Penetratin on translocation efficiency. (A) Confocal fluorescence microscopy of live Hela cells. Cells incubated with the extracellular protein concentration of 2.5 μM for 1 h at 37 °C, with each recombinant protein HD*Antp*, Penetratin and mCherry. Fluorescent signal is displayed by the color of fire from ImageJ, with the maximum projected z stack of 20 confocal slices. Scale bar represents 10 μm. (B) Flow cytometer of Hela cells. Cells incubated with the same condition as (A), with the exception of mCherry at the concentration of 5 μM. Cells were washed three times with 20 U/ml heparin in PBS and trypsinized prio to analysis. (C) Quantification of the cellular uptaking at various concentrations was determined by fluorescent-activated cell sorting (FACS). Data are means of triplicate experiments ± SD.

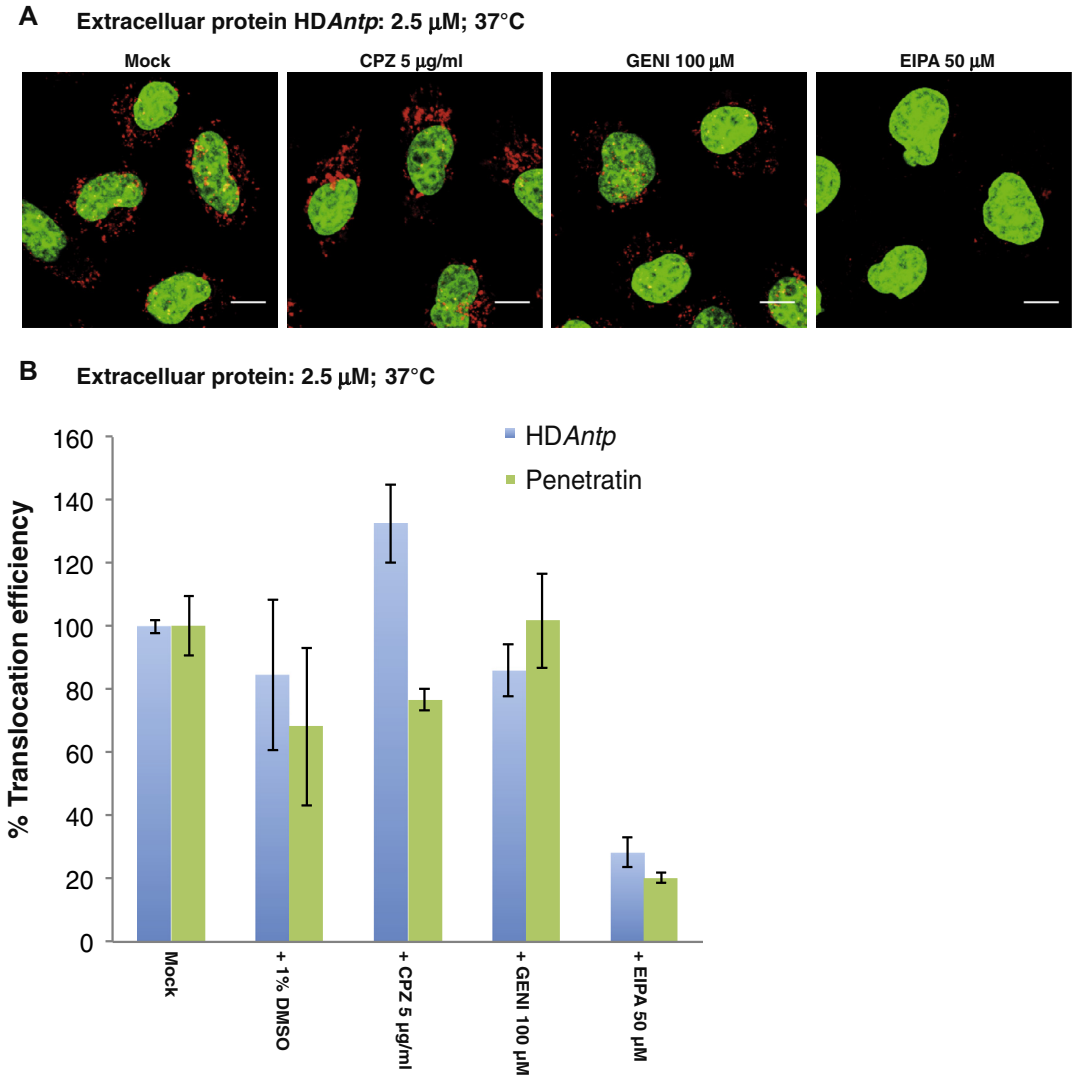

Fig. 3. Pharmacological inhibitors indicating macropinocytosis responsible for protein translocation. (A) Pharmacologically treated Hela H2B::GFP cells were incubated with HD*Antp* at the concentration of 2.5 µM for 1 h, and analyzed by confocal microscopy. Treatments are as follow: untreated (Mock), chlopromazine hydrochloride (CPZ), genistein (GENI), 5-(*N*-Ethyl-*N*-isopropyl) amiloride (EIPA). Images are displayed as a maximum projected z stack of 15 confocal slices. Scale bar represents 10 µM. (B) The effects of endocytic inhibitors and solvent (DMSO) on protein translocation were assayed with FACS. Data are means of triplicate experiments ± SD.

same treatment with an additional trypsinization step prior to FACS analysis. The forward and side scatter gate setting of the FACS ruled out the signal from the dead cells that usually give strong false signals. At the extracellular protein concentration of 2.5 µM, HD*Antp* was able to deliver mCherry with up to 5-fold greater efficiency than Penetratin into Hela cells (Fig. 2B and C). The same pattern was seen with incubation conditions as low as 0.5 µM and as high as 5 µM, although the difference between HD*Antp* and Penetratin narrowed down to 3-fold for the latter (Fig. 2C). Overall, HDAntp exhibited a greater ability to penetrate Hela cells than Penetratin.

To rule out the possibility that the difference is Hela specific, various cell lines were used to compare the efficiency of transduction between HD*Antp* and Penetratin. A higher translocation efficiency of HD*Antp* as compared to Penetratin was observed in mammalian cell lines such as mouse mammary gland derived the normal epithelial cell line NMuMG, human epithelial kidney HEK 293T cells (Sup. 2A), as well as in *Drosophila* S2 cells (Sup. 2B), suggesting the difference in penetration efficiency between the HD*Antp* and Penetratin is cell type independent.

3.3. Macropinocytosis underlying the cellular internalization of HDAntp

As all endocytotic pathways are inhibited at low temperature [14], to assess whether endocytosis is an attribute of protein translocation, Hela H2B::GFP cells were incubated with HD*Antp* at 4 °C and compared to the cells incubated at 37 °C. Confocal microcopy revealed that cold treatment dramatically inhibited the intracellular uptake of HD*Antp* (Sup. 3), indicating endocytic pathways playing a significant role for protein internalization, at least at the specific extracellular protein concentration examined. To determine which endocytic pathway engages in uptake process, we analyzed the uptake efficiency after applying various pharmacological inhibitors to interfere different pathway [15,16]. Chlorpromazine hydrochloride (CPZ) is a cationic amphiphilic drug that inhibits clathrin-mediated endocytosis; Genistein (GENI) is a tyrosine-kinase inhibitor that has been used to inhibit the caveolae-mediated uptake; 5-(*N*-Ethyl-*N*-isopropyl) amiloride (EIPA) is the derivative of amiloride which has been shown to specifically inhibit macropinosome formation [16,17]. With 70% inhibition of internalization,

EIPA was the most efficient inhibitor tested (Fig. 3A and B) when added into the culture medium with either HD*Antp* or Penetratin, indicating that both HD*Antp* and Penetratin recombinant proteins are exploiting macropinocytosis for translocation to a large degree; 100 μM GENI had no effect neither on HD*Antp* nor on Penetratin translocation (Fig. 3A and B); whereas 5 μg/ml CPZ treatment of Hela cells could boost the uptake of HD*Antp* up to 50% (Fig. 3B), but has no significant effects on that of Penetratin (Fig. 3B), which suggesting the potential crosstalk between HD*Antp* translocation and clathrin-mediated pathway, presumably via some compensatory uptake which have been induced by CPZ inhibition.

Macropinocytosis is a highly conserved endocytic pathway by which large volume of extracellular fluid and its contents are internalized into cells, providing cells with a general, non-selective mode of uptake. Therefore the large, heterogeneous vesicles known as macropinosomes formed via this process can be identified by the application of a fluid-phase dye. To visualize macropinocytic uptake, Alexa Fluor® 647-dextran was applied to the medium of cell culture, and the subsequent microscopic examination demonstrated that HD*Antp* co-internalized with fluid phase dye as shown in the Fig. 4.

Altogether, we provide strong evidence that highly positively charged *Antennapedia* homeodomain exploits macropinocytosis to gain access into the cells, and displays higher level of uptake as compared to its widely used third α-helix, "Penetratin". Before, it has been shown that cationic CPPs reach maximal penetrating potency with 8–15 positively charged amino acids and are inhibited by additional positively charged amino acids [9,18], however HD*Antp* (7.8 kDa) that contains up to 18 positively charged amino acids, with other newly identified classes of positively charged proteins, suggests charge distribution and molecular size may also contribute to translocation efficiency.

Macropinocytosis occurs spontaneously in some cells or is triggered in response to growth factor stimulation. As signaling from the plasma membrane is required for actin remodeling to generate mechanical deformation forces, the interaction between positively charged CPPs and negatively charged extracellular heparan sulfate could initially trigger the signaling cascade for fluid phase endocytosis [19]. This would presumably explain why positively charged peptides, polymers, and liposomes are able to penetrate cells. As a fluid phase endocytic pathway, macropinocytosis provides cells with a way to non-selectively internalize large quantities of solute. Thus, macropinocytosis may represent an effective means for drug delivery into cells, particularly for hydrophilic macromolecules. As both of them exploit macropinocytosis to a certain degree, the comparison between "Penetratin" and the *Antennapedia* homeodomain may advance our understanding of the mechanisms of triggering macropinocytotic endocytosis.

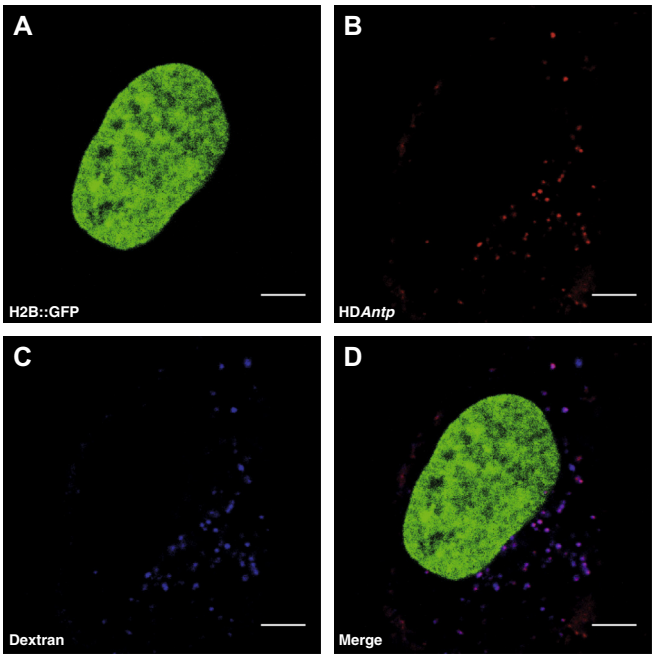

Fig. 4. Internalized HD*Antp* co-localizating with fluid phase Dextran dye. Hela H2B::GFP cells were incubated in the presence of HD*Antp* and fluid-phase dye Dextran Alex-647 at 37 °C for 1hr. Mono-channel for GFP (A), HD*Antp* (B) and Dextran (C), and emerged of each channel. All images are displayed from one confocal slice. Scale bar represents 5 μM.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.062.

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