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Efficient Intracellular Delivery of GFP by Homeodomains of *Drosophila* Fushi-tarazu and Engrailed Proteins

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The 60 amino acid long homeodomain of Antennapedia (Antp), either alone or as a fusion protein with 30–40 amino acid long foreign polypeptides, has been reported to cross biological membranes by an energy- and receptor-protein-independent mechanism. Moreover, the 16 amino acid long third helix of the Antp homeodomain, so-called penetratin, possesses translocation properties when fused to fewer than 100 amino acids as well. These findings led us to study whether such a protein tansduction property is shared by other homeodomains. We report here that homeodomains of two homeoproteins, Fushi-tarazu and Engrailed, are able to transduce a 238 amino acid long green fluorescent protein into cultured cells as efficiently as other well-known protein transduction domains, such as an internal oligopeptide of Tat and penetratin. These findings suggest that such transduction activity of homeodomains might have some physiological roles and that it can be exploited for development of efficient transduction vectors for research use and protein therapy.

Keywords: Homeodomain; Penetratin; Protein Therapy; Protein Transduction Domain.

Introduction

Internalization of exogenous macromolecules by live cells provides powerful tools for studying cellular functions and may be applied for developing new therapeutic approaches. Since the Tat protein from the HIV-1 virus was reported to be the first protein which was able to enter cells when added to the surrounding media (Frankel and Pabo, 1988; Green and Lowenstein, 1988), other proteins, such as the homeodomain of *Drosophila* Antennapedia (Antp) and HSV-1 VP22 transcription factor, have been shown to be internalized by cells in culture as well (Elliott and O'Hare, 1997; Joliot *et al*., 1991). Small regions of these proteins called protein transduction domains (PTDs), 11–34 amino acids in length, were able to cross the lipid bilayer of cells either alone or as a fused form to some polypeptides or oligonucleotides (reviewed in Derossi *et al*., 1998; Lindgren *et al*., 2000; Schwarze and Dowdy, 2000).

The 16 amino acid long third helix of the Antp homeodomain, so-called penetratin, possesses translocation properties comparable to that of the entire homeodomain (Derossi *et al*., 1994). The cellular internalization of homeoproteins, homeodomains, and penetratin occurs at 4 and 37°C and cannot be saturated, suggesting a penetration mechanism that is energy- and receptor-protein-independent (Derossi *et al*., 1994; 1996; Joliot *et al*., 1991). Therefore, the translocation by penetratin is independent of the classical endocytosis pathway and shows such characteristics as high efficiency, non-cell-type specificity, and low toxicity.

There are some examples of homeoproteins (*e.g*., Hoxa-5) where the full-length homeoproteins were shown to be internalized (Chatelin *et al*., 1996). However, except for the Antp homeodomain- and penetrain-polypeptide fusions, no other case of a cell-penetrating fusion protein made of a homeodomain and a foreign polypeptide, has been reported. We investigated whether other homeodomains, as a fused form to heterologous polypetide, are internalized by cells in culture as well, with the ultimate goal of finding out rules which allow homeodomains to traverse cellular membranes efficiently. Two homeodomains of *Drosophila* homeoproteins, Fushi-tarazu (Ftz) and Engrailed (En), with different levels of homology to the Antp homeodomain, were selected for this study. The Antp and Ftz homeodomains are identical at 52 amino acids out of 60 (87%) and the sequence identity at the third helix

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Abbreviation: GFP, green fluorescent protein.

(42–58) is 94%. The En homeodomain is identical to the Antp homeodomain at 30 amino acids (50%) and the sequence identity at the third helix is 76%. Since the sequence identities between Antp and Hoxa-5 at homeodomains and the third helix are 88 and 100%, respectively, the Ftz homeodomain, with a similar level of identity, has a high chance of being internalized. On the other hand, the En homeodomain, with a much lower sequence identity, may provide some information on how much variation is tolerated for a protein tansduction domain.

Here we report that both of the homeodomains of *Drosophila* are able to internalize a fused polypeptide into cultured cells with high efficiency.

Materials and Methods

Construction of expression vectors and production of the fusion protein pTat-GFP was constructed to express the basic domain (amino acids 49–57) of HIV-1 Tat as a fusion with GFP as follows. First, two oligonucleotides were synthesized and annealed to generate a double-stranded oligonucleotide encoding nine amino acids from the basic domain of HIV-1 Tat. The sequences are (top strand) 5′-TAGGAAGAAGCG-GAGACAGCGACGAAGAC-3′ and (bottom strand) 5′-TCGAGTCTTCGTCGCTGTCTCCGCTTCTTCC-3′. The double-stranded oligonucleotide was directly ligated into the NdeI-XhoI digested pET15b (Invitrogen, Carlsbad, CA) in frame with the 6His open reading frame to generate the HisTat expression plasmid, pHisTat. The sequences of the polylinkers cloned into the plasmid were confirmed with a fluorescence-based automated sequencer (model 373A; Applied Biosystems). Next, the complete *gfp* sequence was amplified from plasmid pEGFP-C2 (Clontech) using the polymerase chain reaction (PCR). PCR was performed using *Pfu* DNA polymerase (Clontech). The sense primer was 5′-CTCGAGGT-GAGCAAGGGCGAGGAGCTG-3′ and the antisense primer was 5′-GGATCCTTACTTGTACAGCTCGTCCATGCCG-AG-3′. The PCR product was digested with XhoI-*Bam*HI and subcloned into XhoI-BamHI sites of the pHisTat vector. Similarly, the PCR product digested with XhoI-*Bam*HI was subcloned into XhoI-BamHI sites of pET15b to construct pGFP, which expresses the GFP fusion protein without a basic domain of HIV-1 Tat. Clones with an expected 0.9-kb insert were selected using XhoI-BamHI restriction analysis and were analyzed by sequencing (Sambrook *et al.*, 1989).

pET15b-FtzHD-GFP was constructed by inserting a coding region of the Ftz homeodomain, prepared by PCR amplification, between the His tag and the open reading frame of GFP in pET15b-GFP. pET15b-EnHD-GFP for expressing the En homeodomain-GFP fusion protein was prepared in a similar way. pET15b-penetratin-GFP was constructed by inserting oligonucleotides coding for amino acids 43–58 of the Antp homeodomain. The sequences in the PTDs and at the junctions were confirmed by DNA sequencing. The His-tagged fusion proteins were produced in *E. coli* BL21 and purified under denaturing conditions using a Ni column following instructions provided by Novagen. The eluted protein was mixed well with one volume of glycerol and stored in a deep freezer at −70°C. The concentrations of the purified proteins were measured by Bradford assay.

Internalization of polypeptides and fluorescence microscopy The cultrure conditions of the *Drosophila* S2 cell were described previously (Han, 1996; Baek *et al.*, 1998). The S2 cells, which were ready for splitting, were mixed with four volumes of M3 medium (Sigma) and 0.5 ml of cell suspension was transferred to each well of a 24-well culture plate. A few microliters of the fusion proteins, ranging from 20 to 500 nM, were added to the medium and mixed well. The time, in the range of 0 to about 12 h, spent for incubation of cells in the culture plate before addition of the fusion proteins made little difference. One hour later, the medium was replaced by 100 μl of trypsin without EDTA (Gibco) and incubated at 25°C for 15 min. Four hundred microliters of PBS was added to each well and the cells were resuspended with a micropipet. The cells were transferred to microfuge tubes and spun at 2,000 rpm for 5 min. The cell pellet was washed three times with 500 μl of PBS. The distribution of fluorescence was analyzed using a fluorescence microscope (Olympus).

Western blot Internalization of polypeptides was performed as described earlier except that 60-mm dishes and 5 ml of cell suspension were used instead of 24-well plates and 0.5 ml of cell suspension, respectively. The fusion proteins were added at a final concentration of 200 nM. The remaining steps were performed as described previously except that the volumes were scaled up tenfold. Cell extracts were prepared by mixing harvested cells with one volume of 2 × Laemmli buffer. The same amount of proteins, calculated after the Bradford assay, was loaded and separated on 10% SDS–polyacrylamide gel and the proteins were transferred onto a nitrocellulose membrane (Amersham). The membranes were incubated with 5% BSA in PBS and then with a polyclonal anti-GFP antibody for 1 h at room temperature. After washing, the membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature. Immunoreactive bands were visualized with an ECL plus kit (Amersham) in accordance with the manufacturer's protocol.

Results and Discussion

The recombinant proteins produced and used in this study are schematized in Fig. 1. They are labeled with his tags at the N-terminus to facilitate their purification. For easy detection of internalization, we produced *Aequorea victoria* green fluorescent protein (GFP) fusion proteins by constructing protein expression plasmids where coding regions of PTD (*e.g.*, penetratin, homeodomain, *etc.*,) and GFP were linked in frame. We employed Tat(49-57)-GFP, which was previously shown to enter *Drosophila* S2 cells efficiently, as a positive control for measuring the transduction activities of homeodomains.

The fusion proteins at concentrations of between 20 and 500 nM were added to *Drosophila* S2 cells at 25°C for 1 h and the internalization was measured by

observing the cells with a fluorescence microscope. All the fusion proteins resulted in similar level of fluorescence, which was proportional to the concentration of the fusion proteins added, and typical results at 200 nM are shown in Fig. 2. When the Tat(49–57)-GFP fusion protein was added to the cell culture medium, the fusion protein was internalized and distributed both in the nucleus and the cytoplasm of S2 cells (Fig. 2B). On the other hand, GFP by itself was not internalized under the same experimental conditions (Fig. 2A). The background fluorescence, due to attachment of fusion proteins to the outer surface of the cells, was reduced to a minimum by treating the transduced cells with trypsin. We then monitored the uptake of penetratin-GFP. Penetratin appears to have a rather strict size-dependency, although Tat PTD has been shown to transduce proteins as big as 120 kDa (Schwarze *et al*., 1999). Fusion proteins of fewer than 100 amino acids have always been internalized by penetratin, but, beyond this size, success is not easy to predict (reviewed in Derossi *et al*., 1998). Since GFP consists of 238 amino acids, the penetratin-GFP fusion protein has a certain probability of not being transduced; however, the penetratin-GFP fusion protein was efficiently transduced, suggesting that the compact conformation of GFP might have allowed efficient transduction by penetratin (Fig. 2C). Moreover, both of the homeodomain-GFP fusion proteins, FtzHD-GFP and EnHD-GFP, were internalized by S2 cells as efficiently as the control fusion proteins (Figs. 2D and 2E). Although the Tat fusion proteins have been shown to be transduced efficiently only when the fusion proteins are prepared under denaturing condition, the homeodomain-GFP fusion proteins, prepared under both native and denaturing conditions, were internalized with comparable efficiencies (data not shown).

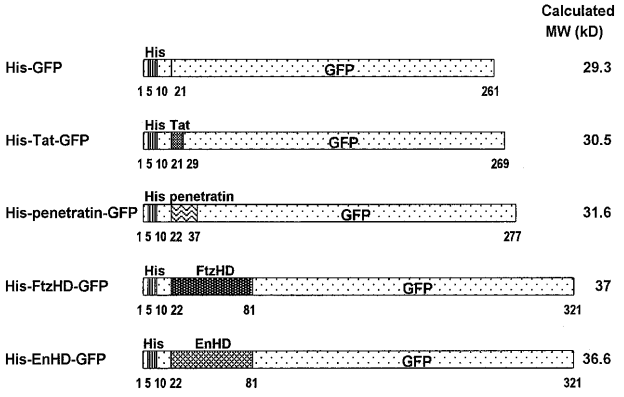

Fig. 1. The PTD-GFP fusion proteins used in protein tranduction studies. The fusion proteins showing the positions of the His tag and each protein transduction domain (PTD) are shown. The numbers below the protein coding regions indicate the number of amino acids from the N-terminus of each fusion protein. All PTDs were cloned into pET15b-GFP plasmid containing the T7 promoter and the *lacO*-operator. The expression is induced by the addition of IPTG. FtzHD: homeodomain of Ftz protein. EnHD: homeodomain of En protein.

To compare transduction efficiencies of different PTD-GFP fusion proteins more quantitatively, we performed Western blot analysis. One nanomole of each of the fusion proteins at a concentration of 200 nM was allowed to be transduced by S2 cells under similar conditions as previously and the relative amount of transduced fusion proteins was quantitated by SDS-PAGE followed by Western blot analysis. The volumes of the cell extracts, prepared from about half of the harvested cells containing the transduced GFP fusion proteins, were adjusted to contain the same amount of proteins in each well. GFP by itself was not transduced at all, as judged from the absence of any GFP-specific band in the blot (Fig. 3). On the other hand, all the fusion proteins were transduced efficiently and recovered from the cells with expected molecular weights (Fig. 3). However, the fusion proteins derived from homeodomains and the Tat fusion protein behaved differently in two ways. First, the penetratin- and

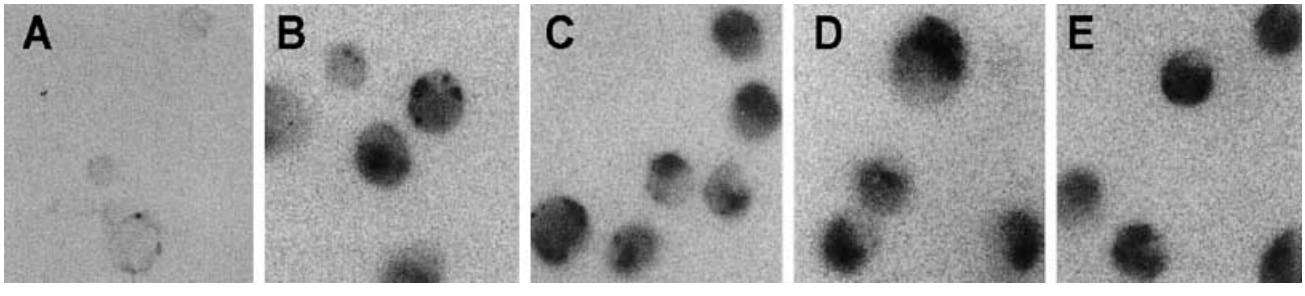

Fig. 2. Transduction of PTD-GFP fusion proteins into cultured S2 cells. Two hundred nanomoles of each of the PTD-GFP fusion proteins shown in Fig. 1 was added to a 24-well plate containing *Drosophila* S2 cells. One hour later, the transduced cells were treated with trypsin, washed several times with PBS, and the distribution of fluorescence was analyzed using a fluorescence microscope. All the PTD-GFP fusion proteins entered S2 cells, whereas GFP did not. Representative fields are shown and the photomicrograph was obtained by printing a dark-field image of the cells. (**A**) GFP. (**B**) Tat-GFP. (**C**) penetratin-GFP. (**D**) FtzHD-GFP. (**E**) EnHD-GFP.

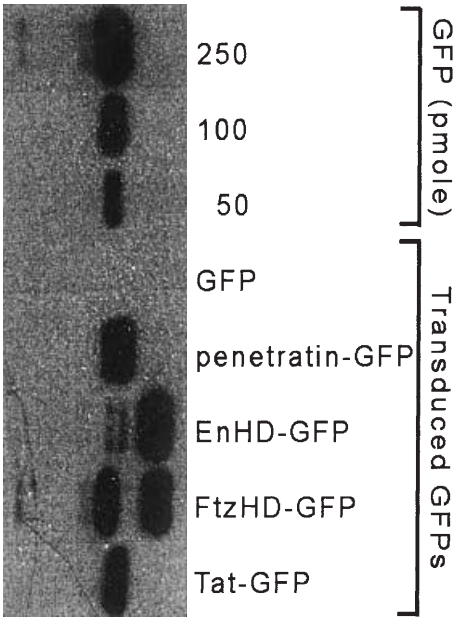

Fig. 3. The PTD-GFP fusion proteins were transduced with similar efficiencies. One nanomole of each of the PTD-GFP fusion proteins at a concentration of 200 nM was added to a 60-mm culture dish containing 5 ml of *Drosophila* S2 cells. One hour later, the transduced cells were treated with trypsin, washed several times with PBS, and the harvested cells were mixed with one volume of 2 × Laemmli buffer. The volumes of the cell extracts, prepared from about half of the harvested cells, were adjusted to contain the same amount of protein in each well. To quantitate the efficiencies of transduction, known amounts of GFP were loaded in the same gel. In all cases, intact PTD-GFP fusion proteins, with expected molecular weights, were recovered from the S2 cell. The PTD regions of two PTD-GFP fusion proteins, penetratin-GFP and FtzHD-GFP, appeared to have been partially removed in the cell.

homeodomain-fusion proteins appear to be transduced with a little higher efficiency than the Tat- GFP protein. Judging from the band intensities, about half or a higher fraction of the penetratin- and homeodomain-fusion proteins added to the medium was transduced, while about one-third of the Tat-GFP protein was transduced. Second, although the Tat-GFP protein was not degraded under the experimental conditions, the other three fusion proteins appear to be degraded in the cell. With less exposure, the thick band observed for penetratin-GFP turned out to be a doublet corresponding to the fusion protein and GFP (data not shown). Since the amino acid residues 2–232, of a total of 238 amino acids in the native protein, were required for the characteristic emission and absorption spectra of native GFP, the lower bands, corresponding to GFP, are likely to have been produced by degradation of the N-terminal PTDs of the three fusion proteins; therefore, the PTDs derived from homeodomains may have a potential to be used as a transient vector which can be removed from the transduced proteins inside the cells. The removal of PTDs may be beneficial since the native forms of cargo proteins, without any fusion or tags, are produced by the fused proteins once inside the cells. However, several points (*e.g.*, the time course of degradation, intracellular stability of the cargo protein and PTD after removal of a PTD, and the effect of removed PTD on cellular physiology) must be studied before attempting the development of transient vectors.

The transduction efficiencies of the two different homeodomains turned out to be comparable, suggesting that the extent of sequence identity between penetratin and a homeodomain has little to do with the transduction efficiency of homeodomains. However, there appears to be a correlation between degradation of PTDs and the sequence identity at the third helix region of homeodomain-derived PTDs. The transduction of both penetratin-GFP and FtzHD-GFP, with a high sequence identity of 94% to the penetratin, resulted in full-length fusion proteins and a GFP protein without PTD in a ratio of about 1:1. On the other hand, EnHD-GFP, with a lower sequence identity of 76% to the penetratin, was only marginally degraded after transduction.

The homeodomains of two homeoproteins, Ftz and En, have been shown to be able to function as PTDs for the first time. This finding implies that the transduction activity may be a common property of diverse homeodomains, although larger numbers of homeodomains have to be tested before making any conclusion. Even though we observed transduction using an artificial fusion protein, this phenomenon is not just an artefact because some native full-length homeoproteins (*e.g.*, Hoxa-5) have been already shown to be transduced (Chatelin *et al.*, 1996). Even though the physiological role of the transduction activity of homeodomains is not clear at this moment, homeodomains can be applied to the development of efficient transduction vectors for hydrophilic macromolecules. The full-length homeodomain is of little use as a transduction vector since it can interfere with the normal gene circuitry of cells, although this property may be exploited in some exceptional cases. On the other hand, no noticeable side effect has been reported for penetratin, which is the third helix of the Antp homeodomain. Therefore, it is of great importance to test whether the third helices of the Ftz and En homeodomains, or even shorter oligopeptide derivatives, retain transduction activity as well. From such a study, it may be possible to develop novel transduction vectors, which are more efficient than commercialized penetratin, for diverse applications, such as research tools and protein therapy.

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