We have recently reported that a 16-amino acid long polypeptide corresponding to the third helix of the DNA binding domain (homeodomain) of Antennapedia, a Drosophila transcription factor, is internalized by cells in culture (Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) J. Biol. Chem. 269, 10444–10450). The capture of the homeodomain and of its third helix at temperatures below 10°C raised the problem of the mechanism of internalization. The present demonstration, that a reverse helix and a helix composed of α-enantiomers still translocate across biological membranes at 4 and 37°C strongly suggests that the third helix of the homeodomain is internalized by a receptor-independent mechanism. The finding that introducing 1 or 3 prolines in the structure does not hamper internalization also demonstrates that the α-helical structure is not necessary. The data presented are compatible with a translocation process based on the establishment of direct interactions with the membrane phospholipids. The third helix of the homeodomain has been used successfully to address biologically active substances to the cytoplasm and nucleus of cells in culture (Théodore, L., Derossi, D., Chassaing, G., Llirbat, B., Kubes, M., J. ordan, P., Chneiwess, H., Godement, P., and Prochiantz, A. (1995) J. Neurosci. 15, 7158–7167). Therefore, in addition to their physiological implications (Prochiantz, A., and Théodore, L. (1995) BioEssays 17, 39–45), the present results open the way to the molecular design of cellular vectors.

Homeoproteins are transcription factors involved in several important biological processes occurring primarily, but not exclusively, during development. The DNA binding domain of these transcription factors is highly conserved and is called the homeodomain. It consists of 60 amino acids arranged in 3 α-helices. Helix 3 is separated from helix 2 by a β turn and is called the recognition helix, because it is involved in the interaction of the homeodomain with specific sites in the large groove of double-stranded DNA (Gehring et al., 1994).

In the course of our studies on the role of homeoproteins in neuronal development we observed that the homeodomain of Antennapedia, a Drosophila homeoprotein, is internalized by cells in culture and, following internalization, is conveyed to the nucleus of cells in culture (Théodore et al., 1995; Troy et al., 1996). A strong incentive to start experiments aimed at understanding its mechanism of translocation of this homeodomain was the demonstration that a reverse helix and a helix composed of D-α-amino acids, in particular when in cells in culture, can adopt an α-helical structure in a hydrophobic environment. We also observed a tendency of the peptide to form dimers and even multimers in the presence of SDS, a phenomenon tentatively attributed to the formation of and association with detergent micelles. In the present study we report new data demonstrating that internalization does not require specific interactions with a chiral receptor (binding site or transporter) or the formation of a charged pore by an α-helical conformation of the peptide.
The cell culture conditions have been described in earlier reports (Lafont et al., 1992). Briefly, small fragments from the cortical-striatal region of the embryonic rat brain between E13 and E15 were incubated in trypsin (0.25%; Life Technologies, Inc.) for 5 min at 

37°C, washed twice in phosphate buffer containing 33 mM glucose, 2 mM glutamine, 10 mM Hepes, pH 7.4, 3 mM NaHCO₃, penicillin 5 units/ml, and streptomycin 5 μg/ml (SFM). The cells were dissociated mechanically, washed three times in SFM, and plated at a concentration of 25,000 cells/cm² on glass coverslips (16-mm diameter) coated with 15 μg/ml α-polyornithine (Sigma). All cultures were in chemically defined medium consisting of SFM supplemented with 0.1% ovalbumin, 100 μg/ml transferrin, 20 nm progesterone, 20 μm putrescine, and 30 nm selenium.

Peptide Internalization and Visualization—When added to cells in culture, peptides were diluted in the chemically defined medium supplemented with 10% fetal calf serum and the following mixture of protease inhibitors: 0.5 mM Pefablock, 1 μg/ml α2-macroglobulin, and 10 μg/ml leupeptin. 2-4 h after the addition of a volume of peptide equal to the volume present in the dishes, the cells were washed three times with the chemically defined medium, fixed for 5 min in 4% paraformaldehyde at room temperature, and then for 5 min at 20°C in ethanol/CH₃COOH(95/5), washed three times in PBS, and incubated for 30 min with fluorescein-linked streptavidin (Amersham Corp.) diluted 1000-fold into PBS plus 10% newborn calf serum (Life Technologies, Inc.). At the end of the incubation the cells were washed three times in PBS, once in water, dried, and mounted in Mowiol for examination.

In some experiments freshly dissociated cells were resuspended in 0.6 ml of PBS or chemically defined medium containing appropriate peptide concentrations plus protease inhibitors. After 2-4 h of incubation with regular gentle shaking, the cells were centrifuged and washed once with 1 ml of PBS plus protease inhibitors and a second time with PBS adjusted to 0.5 mM NaCl. The final cellular pellet was directly resuspended in Laemmli buffer or sonicated to allow for a brief purification of the peptides on streptavidin-agarose (Life Technologies, Inc.). The cells or the beads were boiled and frozen for storage at -80°C or immediately loaded onto a 12%–22% polyacrylamide SDS gradient gel. Following migration, peptides were electrotransferred onto Immobilon (Amersham Corp.) in 25 mM Tris, 192 mM glycine, pH 8.8, the filter was fixed in 4% glutaraldehyde for 15 min, and nonspecific binding sites were blocked by a 2-h incubation in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween 20, and 4% bovine serum albumin. The blots were incubated 1 h at room temperature with streptavidin-peroxidase (Amersham Corp.) in 25 mM Tris, 192 mM glycine, pH 8.8, the filter was fixed in 4% glutaraldehyde for 15 min, and nonspecific binding sites were blocked by a 2-h incubation in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween 20, and 4% bovine serum albumin. The blots were washed several times with the same solution, and revealed with luminol, in accordance with the instructions of the manufacturer (Amersham Corp.).

Confocal Microscopy—Data were obtained with a confocal laser scanning microscope Sarastro 2000 (Molecular Dynamics). Excitation was with an argon ion laser set at 488 nm for fluorescein isothiocyanate excitation, and the emitted light was filtered with an appropriate long pass filter (510 nm). Sections presented were taken approximately at the mid-height level of the cells. Photomultiplier gain and laser power were identical within each experiment.

ELISA Quantification—The cells were incubated with the different peptides as described previously. After several washes with PBS, approximately 5 × 10⁵ cells per condition were plated on ELISA wells. Cells were left to attach for 2 h, fixed overnight in 5% paraformaldehyde at 4°C, permeabilized in methanol, and rinsed three times in PBS. Endogenous alkaline phosphatase was neutralized by incubating the
ELISA plate at 65°C for 1 h. After blocking in 100 mM Tris-HCl, pH 7.4, NaCl 0.3 M, 0.2% Tween, and 5% milk, cells were incubated for 30 min, at room temperature, with alkaline phosphatase-streptavidin (Vectastain ABC kit). Alkaline phosphatase activity was measured spectrophotometrically at 405 nm using a freshly made solution of 1 mg/ml nitrophenyl phosphate (Sigma) in 10 mM diethanolamine (pH 9.5), 0.5 mM MgCl₂.

Electron Microscopy—The cells (2 × 10⁶/ml) were incubated for 2 h at 37°C with the peptides. After several washes with culture medium, the cellular pellets were included in a collagen gel (50% collagen in culture medium), fixed for 30 min at 4°C in PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde, washed in PBS, incubated for 30 min at 4°C in PBS containing 0.3% NH₄Cl, and rinsed overnight at 4°C with PBS. Progressive lowering of temperature was performed in an AFS Reichert (Leica) apparatus. This procedure involves stepwise reductions in temperature (20 to 2°C) as the concentration of the dehydrating agents is increased (30 to 95% ethanol). The infiltration in resin (Lowicryl K4M, Boiziau distribution) was performed at 2°C by progressively increasing the resin concentration and decreasing the percentage of absolute ethanol. Polymerization was carried out at 2°C for 48 h using reflected light from an ultraviolet source. The temperature was slowly increased to reach 20°C. Ultrathin sections from the different blocks were prepared and mounted on 400 mesh Formvar-coated nickel grids. Immunodetection was performed as follows: pretreatment in PBS containing 5% bovine serum albumin for 30 min, washes in 0.1% bovine serum albumin-PBS, incubation for 1 h in PBS containing 0.01% gelatin, 1/200 colloidal gold streptavidin conjugate (15 nm, British Bio Cell International), washes in PBS and H₂O, fixation for 5 min in 1% glutaraldehyde, and washes in H₂O. After air drying, sections were contrasted using 4% uranyl acetate in water and Millonig’s lead acetate-tartrate. The samples were analyzed with a Philips 400 electron microscope.

RESULTS

Peptide Internalization Is Not Receptor-mediated—To investigate whether 43-58 internalization requires a chiral receptor, we synthesized peptides 43-58D and 58-43. Peptide 43-58D has a primary sequence identical to that of 43-58 but is entirely composed of D-enantiomers. Peptide 58-43 is composed of the same amino acids as peptide 43-58, but the order of the amino acids has been reversed, thus leading to a totally different primary structure. Fig. 2, in which the peptides are represented in a α-helical conformation, illustrates that the modifications of the original peptide modify the orientation of the helix but not its amphipathic profile.

Peptide internalization was tested on nerve cells after 2 days of development in vitro. Peptides at a concentration of 22 μM were added for 2 h at 4°C or 37°C. Peptide 41-55, which is not internalized (Derossi et al., 1994), was used as a control. After several washes in PBS, including a final wash in PBS adjusted to 0.5 μM NaCl, the cells were fixed and the presence of biotin revealed with fluorescein isothiocyanate-labeled streptavidin. Fig. 3 illustrates that the 3 peptides, in contrast to...
The peptides 41-55, are internalized by the cells at both temperatures.

To establish that the internalized peptides were not degraded, the peptides retrieved by incubating the cellular extracts on streptavidin-agarose were separated by SDS-PAGE and blotted, and their presence was revealed with streptavidin-peroxidase (see “Materials and Methods” for details). Fig. 4 demonstrates that 58-43 and 43-58D are internalized at 37°C (Fig. 4A) and 4°C (Fig. 4B). All peptides run as monomers (asterisks), dimers, and multimers on an SDS gel. For size comparison, the 11-amino acid long substance P peptide (molecular mass, 1670 Da), aprotinin (molecular mass, 6500 Da), and lysozyme (molecular mass, 14400 Da) were run in parallel. Note that peptide 41-55 is not internalized and that peptide 43-58 is highly sensitive to degradation at 37°C but not at 4°C.

This first series of experiments strongly suggested that peptide internalization is not receptor-dependent. To better establish this point, we compared the localization of 43-58 and 43-58D at the ultrastructural level. Fig. 5 illustrates that the two peptides can be localized within the cells, both in the cytoplasm and in the nucleus. The presence of gold particles in endocytic figures was rarely observed.

Is the α-Helical Structure Important?—To test the importance of the helical structure for internalization, two peptides were synthesized into which were introduced one or three prolines. In Pro50, the glutamine in position 50 was replaced by a proline. In 3Pro, in addition to Gln-50, Ile-45, and Lys-55 were also replaced by proline residues. As illustrated in Fig. 3, introducing one or three prolines into the sequence did not hamper internalization at 4 or 37°C, suggesting that the positions modified are not important and that a helical structure is not mandatory for translocation.

That the peptides were intact after internalization was verified by gel electrophoresis and Western blotting of the peptides retrieved from the cells and purified on streptavidin-agarose. As demonstrated in Fig. 4, the two peptides migrate on the gels with electrophoretic mobilities which indicate that they are intact and suggest that they can form dimeric and multimeric structures.

Although the translocation at 4 and 37°C allows for the addressing of the two peptides to the cytoplasmic compart-
ment, we noted that peptides Pro50 and 3Pro were not conveyed to the nuclei to the same extent as were the other peptides. This latter point is illustrated in Fig. 3. It is shown that, compared to 58-43, which is present throughout the cytoplasm and nuclei of the cells, 3Pro is concentrated in the cytoplasm. Pro50 behaved similarly to 3Pro, whereas the distributions of 43-58 or 43-58D are identical to that of 58-43.

Quantification—The patterns of staining observed after internalization as well as the profiles of the Western blots suggested that all peptides were not internalized with the same efficiency. However, the small size of the peptides and the number of steps necessary to obtain a Western blot precluded the use of the latter approach to compare rigorously the internalization of the different peptides. To estimate better the amount of peptide internalized, an ELISA test was developed (see "Material and Methods" for details). In a preliminary experiment we used the 43-58D, which, because of the D-enantiomers is unlikely to be rapidly degraded, to define an optimal concentration to be used for this analysis. As shown in Fig. 6A, 43-58D internalization (2 h at 37 °C) increases linearly with concentration and does not saturate between 10 and 80 μM.

The comparison between the different peptides used in this study was thus done at a concentration of 40 μM and both at 4 and 37 °C. Similar results were obtained at the two temperatures except for 43-58 which, as already mentioned, is subject to proteolytic degradation at the highest temperature. Fig. 6B illustrates the results of such an experiment, done at 4 °C. It confirms that 41-55 is not internalized. In addition, it suggests that all peptides are internalized with a similar efficiency, except for Pro50, for which the amount recovered inside the cells is significantly and reproducibly higher.

DISCUSSION

The strongest evidence against receptor-mediated endocytosis is the internalization of 43-58D and 58-43. The two peptides are very unlikely to interact specifically with a receptor that would recognize a precise sequence of amino acids (Wade et al., 1990). We cannot, however, entirely exclude the possibility that a receptor or a family of receptors would recognize a general structure conserved between the three polypeptides, in particular a defined organization of hydrophobic and charged amino acids. Even if such interactions exist, the formation of endocytotic coated vesicles is not compatible with internalization at 4 °C. In addition, endocytotic figures were almost never observed at the ultrastructural level.

NMR studies demonstrated (not shown) that in Pro50 the 43-50 domain is in a β-sheet conformation, the α-helix being maintained from residue 51 to residue 58. Although we do not have the corresponding NMR data, it is highly unlikely that the presence of three prolines would be compatible with an α-hel-
Homeodomain Peptide Internalization

...structural features have now permitted the identification of structural features responsible for this unexpected property. Although we do not yet understand the details of the mechanism of translocation, the simple fact of being able to fabricate a D-helix unlikely to be rapidly degraded, or variants (Pro50 and 3Pro) that could address exogenous molecules exclusively to the cytoplasm is of obvious interest for the study of intracellular functions.

A second point of interest is the physiological significance of homeodomain and homeoprotein internalization. Although the internalization and even the nuclear addressing of exogenous proteins has been noted before (Prochiantz and Théodore, 1995; Rubartelli et al., 1990), it is seldom that a systematic study of the structure and mechanisms involved in these processes has been undertaken. It is striking that, in the case of Antennapedia, the structure which is necessary for translocation corresponds to the third helix of the homeodomain. Because this helix is highly conserved among several homeodomains, it is possible that membrane translocation is a property shared by several homeodomains. This latter point is supported by experiments showing that the homeodomains of Engrailed, fushi-tarazu, and Hoxa-5 are internalized (Chatelin et al., 1996).

More interesting is the finding that full-length Hoxa-5 (Chatelin et al., 1996) and Hoxc-8 are also internalized and targeted to the nucleus of cells in culture. Our proposal is that the third helix of a homeodomain could induce the formation of an inverted micelle and thus of a hydrophilic cavity capable to accommodate an entire homeoprotein. It has to be placed in the perspective of the hypothesis that cells may exchange positional information through the local trading of homeoproteins (Chatelin et al., 1995; Prochiantz and Théodore, 1995).

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