Identification of a Signal Peptide for Unconventional Secretion

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Homeoproteins are a class of transcription factors defined by the structure of their DNA-binding domain, the homeodomain. In addition to their nuclear cell-autonomous activities, homeoproteins transfer between cells, thanks to two separate steps of secretion and internalization, which both rely on unconventional mechanisms. Internalization is driven by the third helix of the homeodomain (Penetratin) through a non-vesicular and endocytosis-independent mechanism. In contrast, homeoprotein secretion involves vesicular compartments and requires the presence of a sequence of 11 amino acids (Sec sequence) spanning between the second and third helix of the homeodomain. In this study, we report that the SecPen polypeptide, which combines the two identified domains, Penetratin and Sec, bears all of the necessary information to go in and out of cells. We have analyzed key mechanisms and demonstrated that this peptide can efficiently cross a tight junction epithelium. 

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Biotinylated peptides were synthesized by Neosystem (France) with a biotin residue at the N terminus using the following sequences: Pen (RQIKIWFQNRRMKWKK), PenPro (RQIKIWFQNRRMKWKK), Tat (GRKKRRQRRRP), SecPen (QSLAQELGLNERQIKIFQNNRMRKKWK), and SecTat (QSLAQELGLNERQIKGRKRRQRRRP). N-terminally carboxyfluorescein-labeled peptides were a generous gift of B. Christiaens.

Cell Cultures—MDCK I cells (high resistance) were maintained in DMEM:F-12 (1:1) with 10% fetal calf serum and antibiotics. For peptide internalization, cells were plated on glass coverslips (2.5 × 10^4 cells/cm²) and cultured for 24 h. For Transwell studies, cells were seeded at 1.5 × 10^5 cells on a Transwell filter and analyzed after 72 h. 

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12-mm polystyrene Transwell filter (0.4 μm pore size, Costar) and cultured for at least 5–6 days until full differentiation was achieved (electrical resistance > 2000 Ω/cm2).

Peptide Internalization—Carboxy-fluorescein labeled peptides (20 μM, 25 μl) were incubated with MDCK cells in fresh serum-free DMEM/F-12 medium at the indicated times and temperatures in the presence or absence of 0.5 μM Sytox orange (Molecular Probes). Cells were then washed five times with cold medium and processed for confocal microscopy (TCS-SP2, Leica). When indicated, the cells were briefly washed with cold medium and either immersed in a solution of 0.8% (w/v) trypan blue in culture medium or treated with a solution of 0.05% trypsin/0.02% EDTA (5 min, 4 °C) diluted in cold culture medium. Trypsin digestion was inhibited by the addition of fetal calf serum. Cells were then processed for confocal microscopy.

Biotin-labeled peptides (20 μM) were incubated with MDCK cells in 25 μl of fresh serum-free DMEM/F-12 medium at the indicated times and temperatures in the presence or absence of 0.5 μM Sytox orange (Molecular Probes). The cells were then washed five times with cold medium and incubated with unlabelled avidin (10 μM) for 10 min at 4 °C before fixation. For peptide internalization by MDCK monolayers, biotinylated peptides (100 μM solution in 500 μl of DMEM:F-12) were added in the basolateral chamber of the Transwell. Following incubation, filters were extensively washed with cold medium and incubated with avidin (10 μM in fresh DMEM:F-12) for 10 min at 4 °C before temperature shift.

Biotinylated Peptide-Avidin Complex—For internalization experiments, biotinylated peptides (20 μM) diluted in culture medium were preincubated for 15 min at 20 °C with Alexa 488 streptavidin (1 μg/ml; Molecular Probes) before addition in the culture medium. For Transwell experiments, biotinylated peptides (10 μM) diluted in culture medium were preincubated for 15 min at 20 °C with avidin (10 μM) before addition in the culture medium.

Detection of Biotinylated Peptides—Cells were washed three times with cold medium and fixed in 4% paraformaldehyde (4 °C, 10 min) and permeabilized with 0.1% TX-100 in phosphate-buffered saline (20 °C, 5 min). Biotinylated peptides were detected with Alexa 488-Streptavidin (1 μg/ml) and mounted in DAKO fluorescent mounting medium (DAKO Cytomation). Transwell cultures were labeled with polyclonal anti-ZO-1 (2 μg/ml, Zymed Laboratories Inc.) followed by a Cy5-coupled anti-rabbit antibody (7 μg/ml) (Jackson ImmunoResearch Laboratories).

Peptide Transport Assays through MDCK Monolayers—Peptides diluted in DMEM:F-12 were added in the apical (10 μM, 500 μl) or basolateral (10 μM, 1 ml) chamber. The opposite chamber was filled with avidin (10 μM in DMEM:F-12) to increase peptide trapping in this compartment. When indicated, cells were pretreated 15 min at 37 °C with Brefeldin A (10 μM in DMEM:F-12) before addition of the peptides in Brefeldin A-containing medium. For temperature shift, cells were washed five times with fresh cold DMEM:F-12 medium and incubated with avidin (10 μM, 10 min, 4 °C) before temperature shift. At the end of the experiments, the medium of each chamber was collected and precipitated with 4% trichloroacetic acid.

When analyzed, the cells were subjected to acid wash (20 mM NaAc, 2 × NaCl, 5 min, 4 °C) followed by five washes with fresh DMEM:F-12 medium and resuspended in Laemmli buffer. 1/1.5 × 10^6, 1/50, and 1/4 of donor medium, cell extracts, and recipient medium, respectively, were analyzed by Western blot.

Western Blotting—Protein samples were separated on 22% SDS-PAGE and transferred onto Immobilon P membranes (Millipore). Membranes were fixed with 4% (v/v) glutaraldehyde in water for 15 min and extensively washed three times for 5 min each, saturated 1 h with 4% bovine serum albumin in Tris buffer saline containing 0.2% (v/v) Tween 20. Peptides were revealed with biotinylated-streptavidin peroxidase (1/2000 dilution (Amersham Biosciences)) for 15 min and ECL detection. Luminescence was revealed by autoradiography or analyzed and quantified with Chemismart 5000 Imager (Vilmer-Lourbat).

RESULTS

Visualization of Peptide Internalization—Carboxyfluorescein and biotin conjugates are widely used to monitor protein transduction domain (PTD) internalization, but the use of distinct tags can lead to conflicting results. To compare these two types of conjugates, we first checked the behavior of carboxyfluorescein-linked Penetratin (CF-Pen), because it allows direct visualization on live cells. Plasma membrane integrity was evaluated by the intracellular influx of a cell-impermeant DNA dye (Sytox orange), whereas fluorescence of cell surface-associated CF-Pen was removed by trypsin treatment or quenched by the addition of trypan blue. Following the addition of CF-Pen in the medium of MDCK cell cultures, we observed a clear fluorescent cellular staining both at 4 and 37 °C (not shown). However, co-incubation of Sytox orange together with the peptide reveals a significant intracellular staining by the DNA dye at 37 °C (Fig. 1A), which is more pronounced at 4 °C (Fig. 1B), demonstrating...
Unconventional Secretion Signal Peptide

FIGURE 2. Influence of cargo conjugation on Penetratin internalization. CF-Pen (A and B), biotin-Pen (C and D), or an Alexa 488-streptavidin-Pen complex were incubated with MDCK cells at 37 °C (A, C, and E) or 4 °C (B, D, and F) for 1 h. Extracellular staining was quenched by trypan blue (A and B) or by unlabeled avidin masking before fixation (C and D). In contrast with the temperature-independent internalization of biotin conjugates (C and D), Pen internalization becomes strictly dependent on endocytosis upon conjugation to CF (A and B) or avidin (E and F). Scale bar, 50 μm.

membrane damage. By contrast, neither Penetratin (not shown) nor biotinylated Penetratin induces Sytox orange influx at 37 °C (Fig. 1C) or 4 °C (Fig. 1D), demonstrating that the toxic effect of CF-Pen is due to the addition of the carboxyfluorescein moiety and not to Penetratin itself.

Trypan blue (0.8%) efficiently quenches fluorescein (11) and was thus used to distinguish between extracellular and intracellular staining. The addition of trypan blue following CF-Penetratin incubation modified cellular staining dramatically. At 37 °C, the unquenched fluorescence is associated with vesicles (Fig. 2A) and at 4 °C, no signal is observed at all (Fig. 2B). In contrast, mild trypsin treatment did not significantly alter the cellular staining (not shown), demonstrating that CF-Penetratin internalization at 37 °C is by endocytosis and that live cells do not internalize this compound at 4 °C. We then used biotinylated Pen. To mask cell surface-associated biotinylated peptides, living cells were washed and incubated with an excess of unlabeled avidin that binds extracellular accessible biotin (see “Experimental Procedures”). With this protocol, Penetratin was internalized both at 37 °C (Fig. 2C) and 4 °C (Fig. 2D). Interestingly, coupling fluorescent streptavidin to the biotinylated peptide before its addition to the culture medium shifted the subcellular distribution of the complex toward vesicular structures at 37 °C (Fig. 2E) and fully inhibited its internalization at 4 °C (Fig. 2F). Based on these results, we concluded that conjugation to CF or streptavidin significantly affects the properties of Penetratin. Biotinylated conjugates were thus preferred for our study, despite the fact that their visualization requires fixation. Accordingly, in the following experiments, the indicated peptides will always refer to their biotinylated counterparts.

Internalization of SecPen—The Sec sequence is adjacent to and partially overlaps with the third helix of the homeodomain (Pen). The SecPen peptide thus contains the two sequences but is smaller in size than the sum of the two sequences (Fig. 3A). Fig. 3B illustrates that SecPen internalized at 37 °C is preferentially associated with vesicular structures. At 4 °C, SecPen is still internalized but shows a diffuse distribution, mainly in the cytoplasm (Fig. 3C). The difference in subcellular distributions of Pen and SecPen, in particular at 37 °C, could reflect two non-mutually exclusive situations. First, SecPen could be internalized at 37 °C through classical endocytosis and be present in endocytotic vesicles. Alternatively, it could be internalized by a non-endocytotic mechanism thanks to the Pen sequence and, once in the cytoplasm, could associate with a vesicular compartment. To test the latter hypothesis, we performed temperature shift experiments. SecPen was internalized at 4 °C, and after extracellular biotin quenching with avidin, the temperature was kept at 4 °C (Fig. 3D) or raised to 37 °C for 15 min (Fig.
Following the shift, SecPen distribution changed from diffuse to punctate.

Taken together, these results support the idea that part of the vesicular staining observed at 37 °C can be decomposed into two steps, an addressing to the cytosol, thanks to the Pen sequence and a shift to the vesicles thanks to the Sec sequence. Due to the inhibitory effect of Sec sequence deletion on Engrailed secretion (5, 10), we concluded that these vesicles might be involved in unconventional secretion pathways.

SecPen Secretion—Testing the secretion of SecPen following its internalization through the Penetratin pathway required a model allowing us to distinguish, in the culture medium, the non-internalized peptide from the peptide secreted following an internalization step. To that end, we used the Transwell culture system where MDCK cells grown on a porous filter are cultured until formation of a tight junction epithelium characterized by a high electrical resistance (>2000 ohms/cm²) between the basolateral and the apical sides of the monolayer.

Peptide addition in the basolateral or apical compartment at a 10 µM concentration did not modify the electrical resistance, ruling out a transient opening of the tight junctions (not shown). Following addition in one compartment (donor compartment), secretion was evaluated by peptide accumulation in the opposite compartment (recipient compartment) within 1 h. SecPen accumulated in cells independently of the loading compartment (Fig. 4A) but transport was observed only in the basolateral to apical direction. This result demonstrates that, following internalization, a pool of SecPen is released and that, in MDCK cells, SecPen transport is polarized. Quantitative analysis indicates that, although after 1 h, from 10 nmol of peptide added in the basolateral compartment 3.4 pmol is retrieved in the apical compartment (Fig. 4A). The secretion process, on its own, measured by the ratio between secreted and intracellular peptide is close to 25%.

The contribution of the Sec sequence to secretion was assessed by the analysis of Pen alone. Although the peptide is efficiently internalized by MDCK cells, it is never detected in the opposite compartment (Fig. 4B). To preclude an inhibitory role of nuclear retention on Pen secretion, we used the Gln → Pro variant of Pen (PenPro), which is still internalized but is retained in the cytoplasm (7). As shown in Fig. 4C, this variant is not secreted either.

This series of experiments confirm that Pen and SecPen are internalized by a temperature-independent pathway and that the Sec sequence directs the peptide toward a secretion compartment. They also demonstrate that the SecPen sequence is sufficient for internalization and secretion and that secretion is polarized, at least in MDCK cells.

SecPen Secretion Is Distinct from Internalization and Involves Addressing of the Cytosolic Peptide toward Secretion Compartments—The fact that Pen and SecPen are internalized but that only SecPen is secreted suggests that internalization and secretion obey two different mechanisms. To further verify this point, we used the temperature shift paradigm described above (Fig. 3) to dissociate internalization and secretion. SecPen was placed in the basolateral compartment either at 4 or 37 °C. After 1 h at 37 °C, SecPen is detected in the apical compartment (Fig. 4D). By contrast, no secretion occurs at 4 °C (Fig. 4D), even though SecPen internalization is not inhibited at this low temperature as shown by its detection in the cell extracts (Fig. 4D). This difference of sensitivity to low temperature demonstrates that internalization and secretion obey distinct mechanisms.

Vesicular addressing of a cytosolic pool of SecPen (Fig. 3) does not formally rule out that, in parallel, SecPen internalized by endocytosis at 37 °C could also be transported across the epithelium through vesicular transcytosis. To verify this point, we took advantage of the fact that the addition of avidin to biotinylated Pen blocks its direct translocation across the membranes and directs it to the endocytosis pathway. Trans-epithelial transport of an avidin-SecPen complex was tested in the Transwell model. As shown in Fig. 5A, internalization of avidin-SecPen through endocytosis does not allow the secretion of the complex in the opposite compartment, suggesting that SecPen secretion requires its vesicular addressing from the cytosol. This hypothesis was verified by temperature shift experiments. SecPen was first internalized at 4 °C in the basolateral compartment, the remaining extracellular peptide was complexed with avidin to prevent its translocation, and temperature was raised to 37 °C. After 1 h, SecPen is recovered in the apical compart-
properties of both parts of the chimerical peptide, namely Penetratin and SecTat. When tested in the Transwell system, SecTat did not dramatically increase transferrin transcytosis (not shown), as demonstrated by the absence of Sytox orange uptake (not shown). This illustrates that, although equally efficient in cargo vectorization, Penetratin and Tat PTDs have distinct properties and probably use different entry routes into live cells. These results also clearly illustrate that the presence of a cargo conjugate, even as small as a carboxyfluorescein molecule, can dramatically interfere with PTD properties. Although this might seem anecdotal, it has important consequences on PTD studies and on the inability to predict whether a cargo will be actually internalized following its linkage to a PTD.

We also demonstrated that the Sec domain, necessary for Engrailed secretion, when fused to Penetratin, is internalized by live cells and, following internalization, uses a temperature-dependent mechanism to address the chimerical peptide to vesicular compartments. We used an in vitro model of tight junction epithelium to demonstrate that SecPen is internalized at the level of apical and basolateral membranes but that, following internalization, it is only found in the apical compartment. Our study does not address the issue of peptide recycling (internalization and secretion in the same compartment), as it would require an unambiguous distinction between non-internalized and secreted peptides. We cannot thus rule out that SecPen and/or Penetratin could be secreted and internalized from the donor or the receiver compartment. This caveat must mellow our conclusions, but based on migration profiles on SDS-PAGE, it appears that up to 25% of internalized SecPen is secreted in 1 h without degradation. Quantitative analysis of SecPen transfer from ten independent experiments indicates that, following the addition of 10 nmol of peptide in the basolateral compartment, 3.3 ± 0.7 pmol of peptide are retrieved in the apical compartment after 1 h. In similar conditions, the transcytosis of transferrin is 50-fold less efficient. Previous studies on the passage of PTDs across epithelial monolayers have demonstrated that Tat and Penetratin PTDs are incapable of transcellular transport (13–16) and, moreover, are poorly internalized by confluent MDCK cells (13, 14, 16). In agreement with these observations, we found that neither of these two peptides is transported across the epithelium in our system and that Penetratin internalization is significantly reduced when MDCK cells reach confluence (not shown).

In this study, we have demonstrated that the properties of the Penetratin class of PTDs are modified by conjugation to carboxyfluorescein. CF-Pen alters membrane integrity and, as opposed to Pen, is not internalized at 4 °C but only at 37 °C and through endocytosis. Interestingly, CF conjugation to a non-translocated variant of Penetratin (Trp-48 → Phe-48) (6) or to the Tat PTD has no effect on membrane integrity, as measured by the absence of Sytox orange uptake (not shown). This illustrates that, although equally efficient in cargo vectorization, Penetratin and Tat PTDs have distinct properties and probably use different entry routes into live cells. These results also clearly illustrate that the presence of a cargo conjugate, even as small as a carboxyfluorescein molecule, can dramatically interfere with PTD properties. Although this might seem anecdotal, it has important consequences on PTD studies and on the inability to predict whether a cargo will be actually internalized following its linkage to a PTD.

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The polarity of SecPen trans-epithelial transfer implies that one or several steps of this transfer are polarized. SecPen accu-
mulates throughout the cytoplasm when internalized at 4 °C from either side of the epithelium and is secreted upon temperature shift. It is thus likely that the polarity of SecPen transfer relies on secretion rather than internalization. Interestingly, SecPen-avidin internalized by endocytosis is never transported across a tight junction epithelium, it might be that this in vivo passage is not because of the unique properties of these transduction peptides but to other mechanisms, possibly a transient opening of the blood brain barrier. In conclusion, the identification of this small domain provides a template for the design of vector peptides capable of crossing a tight junction epithelium, however, bearing in mind the possible interference of the transported cargo when fused to the vector.

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Unconventional Secretion Signal Peptide

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