Identification of a signal sequence necessary for the unconventional secretion of Engrailed homeoprotein

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Background: Engrailed-1 and Engrailed-2 are homeoproteins – transcription factors implicated in the morphogenesis of discrete structures. Engrailed proteins have a role in patterning the midbrain–hindbrain region and are expressed in the nuclei of rat embryo midbrain–hindbrain cells. We have previously found that both endogenous and exogenously expressed Engrailed proteins also associate with membrane regions implicated in signal transduction and secretion. Within total membrane fractions, a small proportion of Engrailed – about 5% – is protected against proteinase K proteolysis, suggesting that Engrailed has access to a luminal compartment. Together with our finding that homeodomains and homeoproteins can be internalized by live cells, these observations suggest that Engrailed might act as a polypeptidic messenger. In order to investigate this possibility, we looked to see if Engrailed could be secreted.

Results: Engrailed expressed in COS cells can be recovered in abutting primary neurons and this is dependent on a short sequence in its homeodomain distinct from 'classical' secretion signals. This sequence, which overlaps with the sequence necessary for Engrailed internalization and which is highly conserved among homeoproteins, is the first example of an 'unconventional' sequence necessary for secretion. Less than 5% of total intracellular Engrailed is secreted and there is a correlation between secretion and access to the membrane compartment where the protein is protected against proteinase K.

Conclusions: Our results lend weight to the proposal that Engrailed, and possibly other homeoproteins, might act as intercellular polypeptidic messengers.

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Background

The term homeoprotein defines a class of transcription factors which have been structurally and functionally conserved during evolution [1,2]. Homeoproteins have a very important role during early metazoan development when they participate in the morphogenesis of discrete embryonic territories. Homeoproteins are also implicated in later developmental stages, in particular in the nervous system, as illustrated by the effect of distinct mutations on neuronal morphology [3,4], axonal guidance [5,6] and synaptogenis [7,8]. Homeoproteins bind to specific genomic target sites using a very highly conserved sequence of 60 amino acids, the homeodomain [9]. Homeodomains are composed of three alpha helices; the third helix, also called the recognition helix, binds to specific sequences in the large groove of double-stranded DNA [9].

Because the homeodomain is so highly conserved, we have previously used it as a tool to antagonize homeoprotein–DNA binding in neurons, using transient mechanical disruption of the neuronal membrane to allow internalization of protein added *ex vivo* [10]. In the course of these experiments, we observed that the homeodomain of Antennapedia, a *Drosophila* homeoprotein, was spontaneously internalized by cells in culture and dispatched to both the cytoplasm and nucleus [10,11]. This led us to establish that the third helix of the homeodomain is necessary and sufficient for an 'unconventional' internalization [12] and to propose that there is a previously unidentified translocation mechanism that allows the peptide to gain access directly to the cytoplasm and nuclei of live cells [13–15].

Within the homeodomain, the third helix is the most conserved region, raising the possibility that other homeodomains and some full-length homeoproteins are also internalized by live cells and dispatched to their nuclei. We have demonstrated that this can occur for the homeodomains of Hoxa-5 [16], Engrailed (G. Mainguy, H. Ernø, M. Conradt, V. Blanquet, B. Zevnik, M. Karasawa, F. Vauti, R. Kothary, L. Robel, M.V., A.P. and W. Wurst, unpublished observations) and Ftz (A.J. and A.P., unpublished observations) as well as for full-length Hoxa-5 [16], Hoxc-8 (M.V. and A.P., unpublished observations) and

Engrailed (A.J. and A.P., unpublished observations and this report).

The demonstration that full-length homeoproteins can be internalized led us to consider that, in some circumstances, homeoproteins might have a paracrine activity, which would imply that they are secreted —without the use of the classical signal sequence — and then internalized by other cells. The fact that homeoprotein activity is notoriously cell-autonomous, however, and the current absence of data supporting paracrine activity *in vivo* suggests that, if homeoproteins do have paracrine activity, it cannot be massive or even general. In order to define the key experiments to test our hypothesis, we therefore decided that we first needed both to examine if homeoproteins could be secreted as well as internalized and to understand the mechanisms involved. We would then be able to design experiments in which a given homeoprotein is replaced by a version which is transcriptionally active but deprived of its capacity to be transferred between cells, and examine its effect in the developing animal.

To this end, we have decided to concentrate on Engrailed. The Engrailed homeoproteins, Engrailed-1 (En1) and Engrailed-2 (En2), are first expressed in the anterior neuroepithelium in a domain that will develop into the midbrain–hindbrain territory [17,18]. En1 is also expressed in the spinal cord, in the dermomyotome and in the ventral ectoderm of the limb bud. *En1* is necessary for the development of the midbrain–hindbrain, the sternum and the ventral limb bud [19,20], whereas *En2* mutants only have a subtle midbrain–hindbrain patterning defect [21,22]. In the context of our studies, a main advantage of Engrailed (meaning here En1 and En2 collectively) is the existence of a polyclonal antibody that recognizes the mammalian and avian proteins and of a monoclonal antibody that recognizes only non-mammalian Engrailed, in particular chick En2 (cEn2) [23]. The availability of such antibodies is quite unique and they have been used, in 'mouse-into-chick' grafting experiments, to distinguish the endogenous protein(s) from that expressed in the graft [24–27].

In a previous report [28] we have demonstrated that 5% of cEn2 exogenously expressed in COS cells is present in purified caveolae — a class of membrane and vesicle highly enriched in cholesterol and glycosphingolipids and specialized in signal transduction — and co-localizes with GM1 and caveolin, two caveolar molecular markers. An association with such sphingolipid–cholesterol microdomains was also found for En1 and En2 in the rat midbrain and cerebellum at embryonic day 19 (E19). Furthermore, in COS cells — as in E19 rat embryos — a small and identical proportion of membrane-associated Engrailed was protected against proteinase K, suggesting that both endogenous and exogenous Engrailed can get access to a compartment on a secretory pathway.

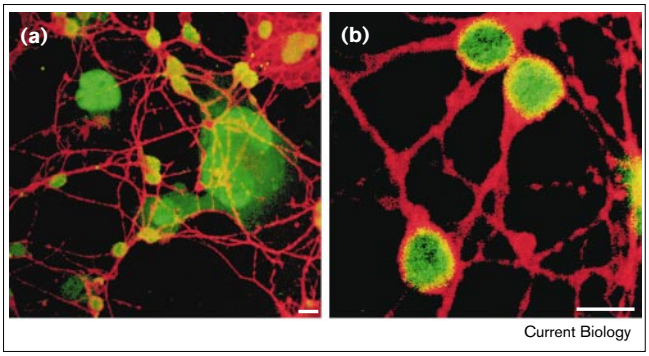
Here, we have used co-cultures of cEn2-expressing COS cells and primary neurons and observed that full-length cEn2 is exported by COS cells and internalized by primary neurons. Moreover, we have identified a sequence that differs from the internalization sequence and is necessary for secretion. Finally, we have shown that the protection against proteinase K of membrane-associated cEn2 is lost in a variant of the protein lacking the sequence necessary for secretion.

Results and discussion

Neurons freshly dissociated from embryonic (E15) rat cortical tissues were co-cultured with COS cells expressing cEn2 protein. After 48 hours, the cultures were fixed and subjected to double immunostaining with the monoclonal anti-cEn2 antibody 4D9 [23] and, as a marker for neuronal cells, a polyclonal anti-NCAM antibody. As shown in Figure 1a, 4D9 stained the nuclei of COS cells but also those of NCAM-expressing rat cortical neurons. Because the 4D9 monoclonal antibody does not recognize the mammalian Engrailed proteins, the staining in cortical neurons cannot correspond to an induction of endogenous Engrailed. High magnification confocal imaging (Figure 1b) demonstrated that the epitope recognized by 4D9 is in the neuronal nuclei. No staining was observed when neurons were cultured with non-transfected COS cells (data not shown).

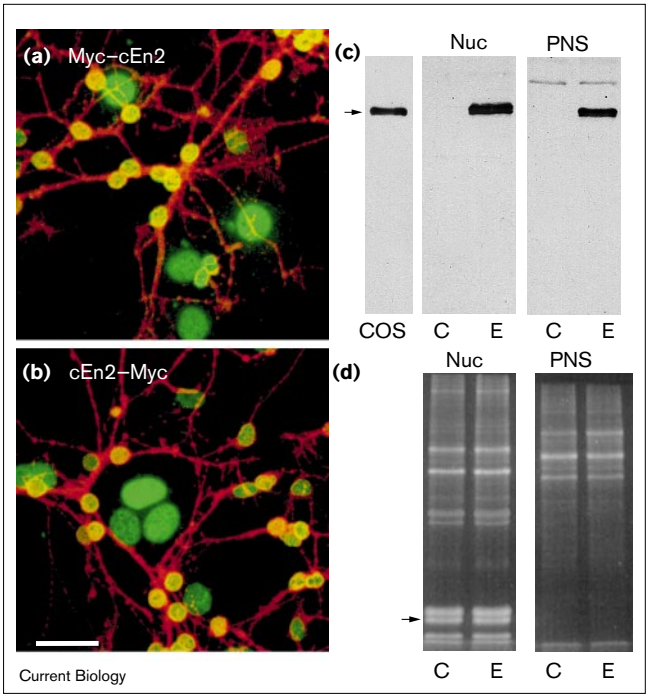
In order to investigate whether full-length cEn2 is secreted and internalized without degradation, a Myc epitope was added to either the amino or carboxyl terminus (to form Myc–cEn2 and cEn2–Myc, respectively). Figure 2a,b illustrates that the anti-Myc antibody stained the nucleus of neurons co-cultured with transfected COS cells expressing either of the tagged proteins. We also analyzed the protein

Figure 1



Transport of cEn2 homeoprotein from COS-7 cells to neurons. COS-7 cells expressing cEn2 were co-cultured with E15 rat embryonic neurons for 48 h. The cultures were fixed and immunostained with the chick-specific monoclonal anti-En antibody 4D9 (green) and a polyclonal anti-NCAM antibody (pk4142; red). Confocal sections at **(a)** low and **(b)** high magnification demonstrate the presence of cEn2 staining in neuronal nuclei. The bar is 10 μm.

Figure 2



Full-length Engrailed protein is transferred between cells. COS-7 cells expressing either **(a)** Myc–cEn2 or **(b)** cEn2–Myc were co-cultured with E15 neurons for 48 h, fixed and immunostained with the anti-Myc (green) and anti-NCAM (red) antibodies. The bar is 10 μm. **(c)** Neurons cultured for 48 h in the vicinity of coverslips bearing COS cells either expressing (E) or not expressing (C) cEn2 were fractionated and analysed by western blot, demonstrating that full-length cEn2 (arrow) was present in cEn2-expressing COS-7 cells (COS) and in the nuclear (Nuc) and postnuclear (PNS) fractions of neurons co-cultured with them. **(d)** Sypro protein staining illustrates that a similar amount of nuclear and postnuclear extracts were loaded; the arrow indicates histones. For comparison, 0.05% (COS), 3.33% (Nuc) and 1% (PNS) of the total volumes were loaded. It is thus estimated that 5% of cEn2 was translocated from expressing to receiving cells within 48 h.

retrieved from neuronal nuclei and postnuclear supernatants (see Materials and methods) and found that it showed no apparent degradation (Figure 2c,d). In the latter experiment, COS cells were cultured on glass coverslips that were introduced into a culture dish containing rat neurons. This procedure allowed an easy and total separation between COS cells and neurons before extraction and western blot analysis using the 4D9 antibody. This series of experiments demonstrates that cEn2 expressed in COS cells can be recovered intact from co-cultured rat neurons.

Pulse chase and experiments done with COS-7 cells in the absence of neurons demonstrated that cEn2 is secreted into the culture medium within an hour of its synthesis and that secreted cEn2 does not pellet at $100{,}000 \times g$ (Figure 3a–c). This suggested that cEn2 is rapidly secreted and freely diffusible in the culture medium and thus that the amount of cEn2 'captured' by co-cultured neurons might be a function of their distance from a cEn2-secreting COS cell. To investigate this, 250 transfected COS cells were scattered on a monolayer of 5×10^5 cortical neurons on a 16 mm diameter dish and the presence of cEn2 in COS cells and neurons was revealed by immunocytochemistry 12, 24 and 48 hours following COS cell addition. As illustrated in Figure 3d, the intensity of nuclear staining in the neurons decreased as the distance to cEn2-expressing COS cells increased, implying that a gradient of Engrailed had formed. The mean surface of Engrailed-positive regions around cEn2-secreting COS cells increased with time (Figure 3e). Because the diffusion fields were approximately circular, we could calculate that the radius of diffusion increased from 28 μm at 12 hours to 80 μm at 24 hours and 100 μm at 48 hours, lengths that correspond to about 3, 8 and 10 neuron diameters, respectively, placed side by side. The latter point is well illustrated in Figure 3f in which a 'chainette' of approximately 10 neuron diameters separates a cEn2-secreting COS cell from a region where cEn2 staining is barely detectable.

The transfer of cEn2 between cells involves two successive steps — secretion and internalization. Preliminary experiments, in which regions upstream of the homeodomain were removed, demonstrated that the fraction of such proteins that was membrane associated upon expression in COS cells was as resistant to proteinase K as membrane-associated wild-type protein [28] (data not shown), suggesting that the homeodomain is important for secretion. Because we already knew that the third helix of the homeodomain, and in particular a WF motif (in single-letter amino acid code; positions 48 and 49 of the homeodomain) within the helix, is important for its internalization and its targeting to the cytoplasm and nucleus [12,29], we substituted the WF motif with an SR doublet (to form cEn2(WF→SR) or the carboxy-terminal tagged version cEn2(WF→SR)–Myc, see Figure 4). In a second mutant, we deleted 11 amino acids upstream of the WF motif (to form cEn2Δ1, see Material and methods and Figure 4). The two mutants and wild-type cEn2–Myc behave differently in COS cells: cEn2Δ1–Myc (Figure 5e,f) was not found in the neurons, whereas cEn2(WF→SR)–Myc (Figure 5c,d) was found at the surface and in the cytoplasm of the neurons but not in their nuclei. Because the WF motif is important for internalization by translocation across the plasma membrane [12,29], the distribution of cEn2(WF→SR)–Myc suggested that cEn2(WF→SR)–Myc is probably secreted but that, as opposed to cEn2–Myc (Figure 5a,b), it does not translocate across the neuronal membrane but is internalized by classical endocytosis.

To evaluate the access of the three proteins to a secretory compartment, the membranes from COS cells transfected

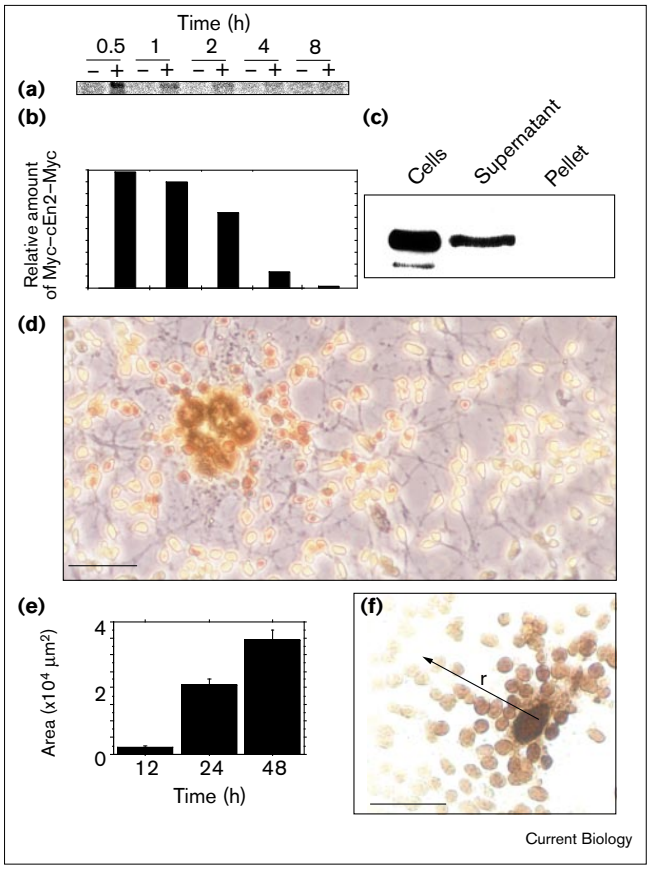


Figure 3

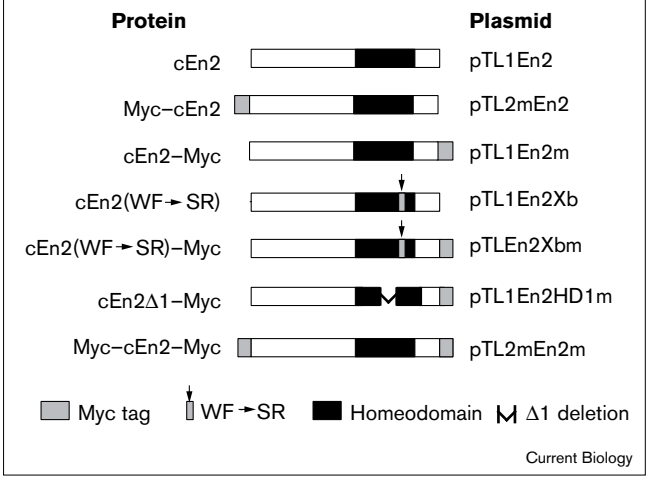
Secretion of cEn2 and formation of a local cEn2 gradient in a neuronal field. **(a,b)** COS cells expressing Myc–cEn2–Myc were cultured for 24 h and the rate of secretion of the expressed protein was followed using pulse–chase analysis and immunoprecipitation with the anti-Myc-tag monoclonal antibody 9E10 as described in Materials and methods: cEn2 is present only in the culture medium of cells producing Myc–cEn2–Myc (+) and not in that of mock-transfected cells (–). The total chase time, following the 30 min pulse of labelling with [^{35}S]methionine, is indicated. (a) Phosphoimaging and (b) its quantification indicate that labeled Myc–cEn2–Myc was progressively diluted as 1 ml aliquots collected from the initial 5 ml tissue culture medium were replaced by fresh medium, thus demonstrating that newly synthesized Myc–cEn2–Myc was secreted within 1 h (30 min labelling and 30 min chase). **(c)** The culture medium from COS cells expressing Myc–cEn2–Myc was separated by centrifugation at $100{,}000 \times g$, as described in Materials and methods. Supernatant and pellet were analyzed for their total Myc–cEn2–Myc content by western-blot analysis (using the 9E10 antibody) together with 1% of the whole cell extract (cells). The absence of Myc–cEn2–Myc in the pellet demonstrates that it is secreted in a soluble form. **(d–f)** COS cells (250) expressing Myc–cEn2–Myc were scattered on 5×10^5 E15 neurons in a 16 mm diameter dish, and the cells were co-cultured for 12, 24 or 48 h, fixed and immunostained with the Myc-specific antibody. (d) Phase-contrast and immunohistochemistry after 48 h demonstrates that Myc–cEn2–Myc is present in the nuclei of differentiating neurons and that the intensity of the staining decreases rapidly as the distance of neurons from the small group of secreting COS cells increases. (e) Quantification of the surfaces covered by positive neurons after 12, 24 and 48 h (analysing 7, 15 and 17 areas, respectively) demonstrates that the gradient is established slowly and does not spread rapidly; Scheffe's test was used to calculate the deviations and all one-tailed p values were < 0.001.
(f) Immunocytochemistry after 48 h of co-culture illustrates that a chainette of approximately 10 contiguous neurons separates the secreting COS cell from the edge of the cEn2-positive domain. The radius (r) of the area of cEn2-positive neurons around the COS cells is indicated by an arrow. The bar is 40 µm. (a–f) In this set of experiments, doubly tagged cEn2 protein (Myc–cEn2–Myc; see Figure 4) was used in order to increase the sensitivity of detection.

with plasmids encoding the proteins were purified and treated with proteinase K. Because the Δ1 deletion precludes recognition by available polyclonal or monoclonal anti-Engrailed antibodies, cEn2Δ1–Myc was always visualized using the anti-Myc antibody. Analysis of the controls corresponding to cEn2Δ1–Myc and cEn2(WF→SR) — cEn2–Myc and cEn2, respectively — also demonstrated that the presence of the Myc epitope did not change the properties of Engrailed proteins, as wild-type and Myc-tagged cEn2 behaved similarly (Figure 6). The three proteins — wild-type cEn2 (or cEn2–Myc), cEn2(WF→SR) and cEn2Δ1–Myc — co-purify with a membrane fraction on a saccharose cushion (Figure 6), but upon proteinase K treatment, only cEn2 (or cEn2–Myc) and cEn2(WF→SR), but not cEn2Δ1–Myc, are protected. Although the proportion of protein protected is low — between 5 and 10% of the membrane-associated proteins — the protection does seem authentic as, firstly, none of the nuclear fraction is protected and, secondly, protection is fully lost after membrane permeabilization. This strongly suggests that the Δ1 domain, or part of this domain, participates in the targeting of cEn2 into a secretory compartment.

The secretion of proteins devoid of signal sequence has been shown in the case of the TAT transcription factor of the human immunodeficiency virus [30], fibroblast growth factor (FGF)-1 and FGF-2 (reviewed in [31]), lactoferrin [32] and the VP22 herpes virus protein [33]. It is noteworthy both that in the case of the TAT protein from an equine virus a structure very similar to that of a homeodomain has been proposed [34] and that all these proteins, following secretion and internalization, gain access to the cell nucleus. We have previous demonstrated that the third helix of the homeodomain is necessary and sufficient for internalization and proposed a mechanism for translocation [13–15]. In contrast, we have not analysed whether the sequence removed by the Δ1 deletion — which encompasses the end of the second helix, the beginning of the third helix and the turn between the two helices — is sufficient for secretion, nor do we know the mechanism that is involved.

Engrailed proteins are the first in which sequences necessary for both unconventional secretion and unconventional internalization have been identified, and it is noticeable that these sequences are highly conserved among homeoproteins. The fact that, in the case of cEn2, the sequences

Figure 4

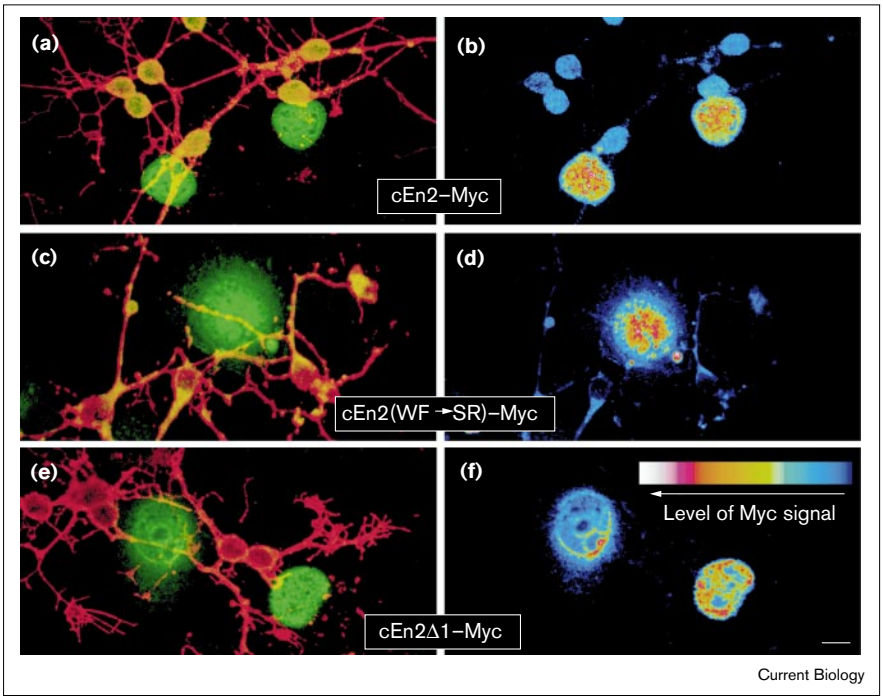


Each of the proteins we have expressed in COS cells is depicted; the plasmids encoding them are also indicated. The Δ1 deletion removes the amino acid sequence QELGLNESQIK, which is upstream of the WF motif.

responsible for internalization by translocation and for unconventional secretion are different suggests that the two events rely on separate mechanisms. In a previous report, we have shown that exogenously expressed Engrailed associates with caveolae in COS cells and that endogenous Engrailed associates with sphingolipid–cholesterol microdomains [28]. Sphingolipid–cholesterol microdomains are specialized in signal transduction and cycle between the plasma membrane and different luminal compartments [35]. Treatments interfering with the cellular content of cholesterol provoke the passage of caveolin, a caveolae scaffolding protein, into the lumen of the endoplasmic reticulum where it is protected against proteolytic enzymes [36]. The sequence necessary for cEn2 secretion overlaps with a domain that has striking similarities to a sequence within caveolin [28], suggesting that cEn2 secretion might involve its translocation into the lumen either of sphingolipid–cholesterol microdomains or of another compartment, for example the endoplasmic reticulum.

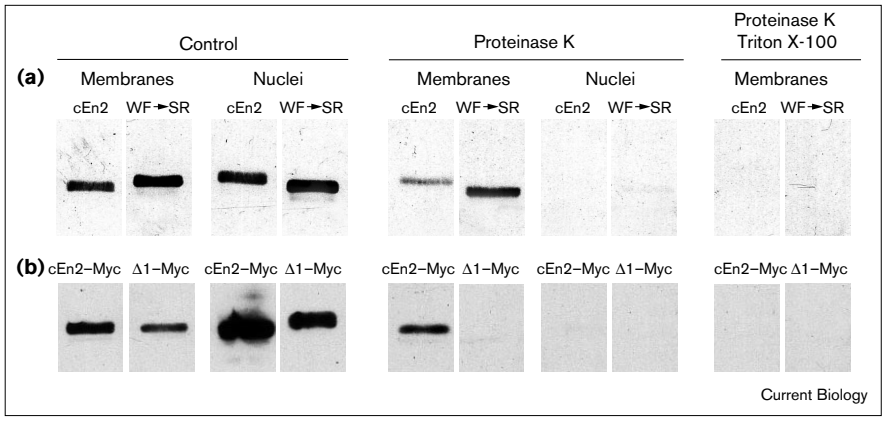
Our results show that cEn2 is transferred between COS-7 cells and co-cultured neurons and we do not yet know whether this reflects a normal event. We propose, however, that such transfer might occur *in vivo* on the basis of the following observations: firstly, secretion and protection against proteinase K are correlated; secondly, endogenous rat En1 and En2 are associated with sphingolipid–cholesterol microdomains *in vivo*; and, thirdly, a fraction of membrane-associated En1 and En2 is protected against proteinase K *in vivo* [28]. We estimate that, *in vitro*, less than 5% of cEn2 gains access to a secretory compartment and is actually secreted. This proportion is in good accordance with the amount of rat En1 and En2 associated with a membrane fraction and protected against proteolysis *in vivo*, in a situation in which the cells synthesize physiological amounts of Engrailed. Determining the

Figure 5



The Δ1 deletion blocks secretion. COS cells expressing either **(a,b)** cEn2–Myc, **(c,d)** cEn2(WF→SR)–Myc or **(e,f)** cEn2Δ1–Myc were co-cultured with E15 rat embryonic neurons for 48 h. After double immunostaining for the Myc tag (green) and NCAM (red), we took a confocal section and looked at (a,c,e) both signals by superposition and (b,d,f) the Myc-specific signal alone using the pseudocolored linear scale shown in (f). The bar is 10 μm.

Figure 6



Engrailed secretion is correlated with its resistance to proteinase K. We expressed **(a)** cEn2 and cEn2(WF→SR) or **(b)** cEn2–Myc and cEn2Δ1–Myc in COS-7 cells for 48 h and we then analysed their presence in membrane and nuclear fractions. For proteinase K treatment, the amount of extracts loaded was (a) 8-fold or (b) 40-fold that of non-treated membranes. The proportion of cEn2 or cEn2(WF→SR) in the membrane fraction protected against proteinase K was estimated at between 3 and 10%, depending on the experiment. Note the absence of protection in the nuclear fraction and the loss of protection in membranes treated with detergent (Triton X-100).

precise level of Engrailed that is secreted, given that it is such a small amount, awaits very refined measurements.

The results presented in Figure 3 also suggest that, in a situation in which cEn2 diffuses freely, the radius of diffusion is no longer than about 10 times the diameter of a neuron, even after it has been expressed for 48 hours, and that the radius of diffusion in fact reaches a plateau between 24 and 48 hours. It is thus very likely that if Engrailed is secreted in a physiological situation, its diffusion would be even more limited. As a result, only the cells in the immediate proximity of the Engrailed source would capture the protein, and this would be in such small amounts that one would not necessarily expect to see a dramatic change in phenotype but, rather, a modification of transcription of a limited number of Engrailed transcriptional targets. We speculate that this might act as a mechanism to establish a border between abutting territories or to mediate the recognition of cells for cells of a different topological origin.

The transfer of homeodomain protein to adjacent non-transcribing cells *in vivo* has been reported in plants: KNOTTED-1, a plant homeoprotein synthesized in the maize vegetative shoot apex, translocates into one row of L1 epidermal cells that do not express its mRNA [37]. KNOTTED-1 is the first homeoprotein reported to have an *in vivo* paracrine activity. Indeed, its translocation into the L1 cells involves plasmodesmata, a specialized structure found only in plants. It seems possible that animals may have developed other molecular mechanisms to achieve a similar function. In this context, it is noteworthy that the homeoprotein Emx-1 is targeted into nuclei but also into the axons of olfactory neurons [38]. Because sphingolipid–cholesterol microdomains are found primarily in the axonal compartment, the transport of Emx-1 into the axons suggests that the observations about Engrailed reported by us in this report and previously [28] might extrapolate to other homeoproteins and to specific *in vivo* situations.

Conclusions

By co-culturing cEn2-expressing COS cells and primary neurons, we have shown that full-length cEn2 is exported by COS cells and internalized by primary neurons. We have identified a sequence in Engrailed that differs from its internalization sequence and that is necessary for its secretion and the protection against proteinase K of the fraction of cEn2 that is membrane associated.

The identification of a sequence necessary for the secretion and protection against proteinase K of Engrailed might be important for the following reasons. Firstly, a sequence necessary for the export of a protein devoid of a 'classical' export signal sequence has not been identified previously. Secondly, cEn2 export by COS cells is correlated with cEn2 protection against proteinase K — a protection that has also been reported for cEn2 in embryonic tissues. Thirdly, the small proportion of Engrailed protein that is present in a compartment compatible with secretion — both in a physiological and a non-physiological situation — and that is, at least in our COS cell system, secreted, suggests that if Engrailed secretion does occur physiologically, it might not be easy to trace and might not lead to an obvious non-autonomous phenotype as classically defined by the embryologists.

Finally, we hope to exploit our present understanding of the mechanisms involved in the import and export of Engrailed to annihilate its paracrine activity without modifying its transcriptional activity: such an experiment will be a first and indispensable step to evaluate the physiological relevance of our observations.

Materials and methods

Plasmids and proteins

The different proteins used in this report are described in Figure 4. All plasmids are derived from pTL1cEn2 [28], in which protein expression is under the control of the SV40 promoter. The Myc–cEn2 and cEn2–Myc proteins have a Myc-tag sequence (EQKLISEEDL) [39] at

the amino and carboxyl terminus, respectively. The cEn2(WF→SR) protein bearing the substitution of the amino acids 247–248 (WF) with an SR motif was produced by site-directed mutagenesis. The deletion in cEn2Δ1 includes amino acids 235 to 245 (QELGLNESQIK). In cEn2(WF→SR)–Myc and cEn2Δ1–Myc proteins, a Myc tag was added at the carboxyl terminus of the proteins. Plasmids and details concerning the constructions are available on request.

Cell culture and electroporation

All cells were grown in modified Dulbeccos' modified Eagles medium/F12 medium (1/1) [40] supplemented with 10% fetal calf serum (FCS) to give complete medium. For electroporation, COS-7 cells ($1–4 \times 10^6$ in 300 µl of complete medium) mixed with 10–20 µg DNA were placed in a 0.4 cm gap electrode cuvette, electroporated (170 V, 950 µF), kept for 10 min at room temperature, washed and pelleted ($200 \times g$, 5 min) before culture. Transfection efficiency was between 50 and 80% of the cells. Rat embryonic neurons were prepared as described [41] with slight modifications. Briefly, tissues freed of meninges were incubated in trypsine–EDTA (0.25% trypsine, 1 mM EDTA; 10 min) and the cells mechanically dissociated in phosphate-buffered saline (PBS) containing 33 mM glucose, 30 µg/ml DNase I (Sigma) and 10% FCS. Dissociated cells were washed three times in PBS containing 33 mM glucose and seeded at the densities indicated in the text.

Immunocytochemistry

COS-7 cells (10^4) were co-cultured with freshly dissociated rat E15 cortical neurons (5×10^4) on 1.6 cm diameter glass coverslips coated with 15 µg/ml polyornithine (Sigma) and 1 µg/ml laminin (Sigma) in complete medium supplemented with DNase I (10 µg/ml). After 12, 24 or 48 h the cells were washed twice in PBS and fixed in ethanol : acetic acid (90:10) for 10 min at –20°C followed by a brief incubation in cold methanol. After drying, immunostaining was performed as previously described [42]. Engrailed protein was detected using the monoclonal antibodies 4D9 or 9E10 (for Myc-tagged constructs) and biotinylated anti-mouse antibody (1/200, Vector ABC-kit) plus streptavidin horseradish peroxidase (1/100, Vector ABC-kit) or streptavidin–FITC (1/800, Vector). For double immunostaining, the anti-NCAM antibody (gift of Christo Goridis) was revealed with Cy3 anti-rabbit antibodies (1/400, Jackson Laboratories). Mounting was in Vectashield (Vector) and all images were obtained by confocal laser microscopy (Molecular Dynamics 1000). Preparations were scanned with appropriate filters (600 nm and 530DF30 nm) and a Gaussian filter was applied to the original data. Pseudocolor images were obtained with an unmodified linear look-up table.

Western-blot analysis

Freshly dissociated rat E16 cortical neurons (10^7) were cultured in a 10 cm diameter plastic petri dish coated with 15 µg/ml polyornithine. Freshly electroporated COS-7 cells (5×10^4) seeded on glass coverslips coated with 15 µg/ml polyornithine were carefully added at the periphery of the petri dish 12 h later (10–12 coverslips per dish; cells facing up), and sealed with a collagen drop. After 48 h, the coverslips were removed and the neurons were lysed for 15 min a 4°C in PBS plus 1 mM Ca^{2+}, 1 mM Mg^{2+}, 0.5% NP40, and protease inhibitors. Nuclear and postnuclear fractions separated by centrifugation (15 min, $2000 \times g$, 4°C) were resuspended in Laemmli buffer. COS-7 cells on the coverslips were collected by trypsin treatment and directly resuspended in Laemmli buffer. Proteins in the samples were separated by SDS–PAGE (12%), stained (SYPRO 1/5000 in blotting buffer) and transferred onto nitrocellulose (BAS 85, Schleicher & Schuell). Immunological detection was performed as described [28]. To determine if secreted Myc–cEn2–Myc was secreted in a soluble form, 10^6 COS cells expressing Myc–cEn2–Myc were cultured in complete medium. After 24 h, the cells were washed twice, incubated for 30 min in medium containing 0.2% FCS, 25 µg/ml heparin and 10 µg/ml DNase I, washed and incubated for a further 3.5 h with the same medium supplemented with protease inhibitors. The medium was cleared by low speed centrifugation ($6000 \times g$, 10 min) and the supernatant was centrifuged for 35 min at $100{,}000 \times g$.

Pulse-chase experiments

COS cells (10^6) expressing Myc–cEn2–Myc were cultured for 24 h on polyornithine-coated 60 mm diameter culture dishes. After washing, cells were preincubated for 20 min in methionine-free medium plus 0.2% FCS, 25 µg/ml heparin and 10 µg/ml DNAse I. Labeling was performed in 5 ml of the same medium containing 1 mCi [^{35}S]methionine (1000 Ci/mmol; Promix, Amersham) for 30 min. Chase medium (5 ml) containing 10 mM methionine, 25 µg/ml heparin and protease inhibitors was added and 1 ml aliquots were collected at the indicated times and replaced by 1 ml fresh medium. All samples were immunoprecipitated as described [40] using the 9E10 anti-Myc antibody. Mock transfected cells were used as a control and the amount of protein immunoprecipitated was quantified by phosphoimaging.

Proteolytic treatments

Membrane fractions and nuclei were prepared as described previously [28]. Each fraction was divided in three parts, and treated for 1 h at 4°C as indicated (100 µg/ml proteinase K, 0.2% Triton X-100). Treatments were stopped by TCA precipitation.

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References

1. Krumlauf R: **Hox genes in vertebrate development**. *Cell* 1994, **78:**191-201.
2. Akam M: ***Hox* and *HOM*: homologous gene clusters in insects and vertebrates**. *Cell* 1989, **57:**347-349.
3. Doe CQ, Hiromi Y, Gerhing WJ, Goodman CS: **Expression and function of the segmentation gene fushi tarazu during *Drosophila* neurogenesis**. *Science* 1988, **239:**170-175.
4. Doe CQ, Scott MP: **Segmentation and homeotic gene function in the developing nervous system of *Drosophila***. *Trends Neurosci* 1988, **11:**101-107.
5. Le Mouellic H, Lallemand Y, Brûlet P: **Homeosis in the mouse induced by a null mutation in the Hox-3.1 gene**. *Cell* 1992, **69:**251-264.
6. Tiret L, Le Mouellic H, Maury M, Brûlet P: **Increased apoptosis of motoneurons and altered somatotopic maps in the brachial spinal cord of Hoxc-8-deficient mice**. *Development* 1998, **125:**279-291.
7. White JG, Southgate E, Thomson JN: **Mutations in *Caenorhabditis elegans unc-4* gene alter the synaptic input to ventral cord motor neurons**. *Nature* 1992, **355:**838-841.
8. Miller DM, Shen MM, Shamu CE, Bürglin TR, Ruvkin G, Dubois ML, *et al.*: ***C. elegans unc-4* genes encodes a homeoprotein that determines the pattern of synaptic input to specific motor neurons**. *Nature* 1992, **355:**841-845.
9. Gehring WJ, Qian YQ, Billeter M, Furukubo-Tokunaga K, Schier AF, Resendez-Perez D, *et al.*: **Homeodomain–DNA recognition**. *Cell* 1994, **78:**211-223.
10. Joliot A, Pernelle C, Deagostini-Bazin H, Prochiantz A: **Antennapedia homeobox peptide regulates neural morphogenesis**. *Proc Natl Acad Sci USA* 1991, **88:**1864-1868.
11. Joliot AH, Triller A, Volovitch M, Pernelle C, Prochiantz A: **α-2,8-polysialic acid is the neuronal surface receptor of Antennapedia homeobox peptide**. *New Biol* 1991, **3:**1121-1134.
12. Derossi D, Joliot AH, Chassaing G, Prochiantz A: **The third helix of Antennapedia homeodomain translocates through biological membranes**. *J Biol Chem* 1994, **269:**10444-10450.
13. Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, Prochiantz A: **Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent**. *J Biol Chem* 1996, **271:**18188-18193.
14. Derossi D, Chassaing G, Prochiantz A: **Trojan peptides: the penetratin system for intracellular delivery**. *Trends Cell Biol* 1998, **8:**84-87.
15. Prochiantz A: **Getting hydrophilic compounds into cells: lessons from homeopeptides**. *Curr Opin Neurobiol* 1996, **6:**629-634.

16. Chatelin L, Volovitch M, Joliot AH, Perez F, Prochiantz A: **Transcription factor Hoxa-5 is taken up by cells in culture and conveyed to their nuclei.** *Mech Dev* 1996, **55:**111-117.
17. Davis CA, Noble-Topham SE, Rossant J, Joyner AJ: **Expression of the homeobox containing gene En-2 delineates a specific region of the developing mouse brain**. *Genes Dev* 1988, **2:**361-371.
18. Gardner CA, Darnell DK, Poole SJ, Ordahl CP, Barald KF: **Expression of an *engrailed*-like gene during development of the early embryonic chick nervous system**. *J Neurosci Res* 1988, **21:**426-437.
19. Wurst W, Auerbach AB, Joyner AL: **Multiple developmental defects in *Engrailed -1* mutants mice: an early mid-hind-brain deletion and patterning defects in forelimbs and sternum**. *Development* 1994, **120:**2065-2075.
20. Loomis CA, Harris E, Michaud J, Wurst W, Hanks M, Joyner AL: **The mouse Engrailed-1 gene and ventral limb patterning**. *Nature* 1996, **382:**360-363.
21. Joyner AL, Herrup K, Auerbach BA, Davis CA, Rossant J: **Subtle cerebellar phenotype in mice homozygous for a targeted deletion of the En-2 homeobox**. *Science* 1991, **251:**1239-1243.
22. Millen KJ, Wurst W, Herrup K, Joyner A: **Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse *Engrailed -2* mutants**. *Development* 1994, **120:**695-706.
23. Patel NH, Martin BE, Coleman KG, Poole SJ, Ellis MC, Kornberg TB, Goodman CS: **Expression of engrailed proteins in arthropods, annelids, and chordates.** *Cell* 1989, **58:**955-968.
24. Wassef M, Bally CL, Alvarado MRM: **Regional specification during cerebellar development.** *Perspect Dev Neurobiol* 1993, **1:**127-32.
25. Bally-Cuif L, Wassef M: **Ectopic induction and reorganization of Wnt-1 expression in quail/chick chimeras**. *Development* 1994, **120:**3379-3394.
26. Millet S, Avarado-Mallart RM: **Expression of the homeobox-containing gene En-2 during the development of the chick central nervous system**. *Eur J Neurosci* 1995, **7:**777-791.
27. Martinez S, Marin F, Nieto MA, Puelles L: **Induction of ectopic engrailed expression and fate change in avian rhombomeres: intersegmental boundaries as barriers**. *Mech Dev* 1995, **51:**289-303.
28. Joliot A, Trembleau A, Raposo G, Calvet S, Volovitch M, Prochiantz A: **Association of engrailed homeoproteins with vesicles presenting caveolae-like properties**. *Development* 1997, **124:**1865-1875.
29. Le Roux I, Joliot AH, Bloch-Gallego E, Prochiantz A, Volovitch M: **Neurotrophic activity of the Antennapedia homeodomain depends on its specific DNA-binding properties**. *Proc Natl Acad Sci USA* 1993, **90:**9120-9124.
30. Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, *et al.*: **Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation**. *J Virol* 1993, **67:**277-287.
31. Prochiantz A, Théodore L: **Nuclear/growth factors**. *Bioessays* 1995, **17:**39-45.
32. He J, Furmanski P: **Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA**. *Nature* 1995, **373:**721-724.
33. Elliott G, O'Hare P: **Intercellular trafficking and protein delivery by a herpesvirus structural protein**. *Cell* 1997, **88:**223-233.
34. Rösch P, Willbord D: **Is EIAV Tat protein a homeodomain?** *Science* 1996, **272:**1672.
35. Parton RG: **Caveolae and caveolins**. *Curr Opin Cell Biol* 1996, **8:**542-548.
36. Smart EJ, Ying Y-S, Donzell WC, Anderson RGW: **A role for caveolin transport of cholesterol from endoplasmic reticulum to plasma membrane**. *J Biol Chem* 1996, **271:**29427-29435.
37. Lucas WJ, Bouché-Pillon S, Jackson DP, Nguyen L, Baker L, Ding B, Hake S: **Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata**. *Science* 1995, **270:**1980-1983.
38. Briata P, Di Blas E, Gulisano M, Mallamaci A, Iannone R, Boncinelli E, Corte G: **EMX1 homeoprotein is expressed in cell nuclei of the developing cerebal cortex and in the axons of the olfactory sensory neurons**. *Mech Dev* 1996, **57:**169-180.
39. Evan GI, Lewis GK, Ramsay G: **Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product.** *Mol Cell Biol* 1985, **5:**3610-3616.
40. Rousselet A, Fetler L, Chamak B, Prochiantz A: **Rat mesencephalic neurons in culture exhibit different morphological traits in the presence of media conditioned on mesencephalic or striatal astroglia**. *Dev Biol* 1988, **129:**495-504.
41. Chamak B, Prochiantz A: **Influence of extracellular matrix proteins on the expression of neuronal polarity**. *Development* 1989, **106:** 483-491.
42. Allinquant B, Moya KL, Bouillot C, Prochiantz A: **Amyloid precursor protein in cortical neurons: coexistence of two pools differentially distributed in axons and dendrites and association with cytoskeleton**. *J Neurosci* 1994, **14:**6842-6854.

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