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Association of Engrailed homeoproteins with vesicles presenting caveolae-like properties

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SUMMARY

We report here that the homeoproteins Engrailed-1 and Engrailed-2 are present in specific non-nuclear subcellular compartments. Using electron microscopy, we observed that chick-Engrailed-2 expressed in COS-7 cells associates with membrane fractions that are characterized as caveolae. This characterization is based on morphological, biochemical and immunological criteria such as, in particular, the absence of clathrin coat and the presence of caveolin and cholera toxin-binding sites. These data are fully confirmed by subcellular fractionation experiments, which demonstrate that transfected chick-Engrailed-2 is present in low density membrane fractions that are resistant to Triton X-100, enriched in caveolin and solubilized by the addition of a cholesterol-binding detergent, a set of properties highly characteristic of caveolae. The association of Engrailed-2 with specific membrane fractions observed after transfection in COS-7 cells is also observed for endogenous Engrailed-1 and Engrailed-2 expressed at late embryonic stages in the cerebellum and posterior mesencephalon of the rodent. Indeed, the two proteins are present in membrane fractions that bear all the characteristics of microdomains or caveolae-like domains, i.e. Triton X-100 resistance, saponin solubilization, low density on sucrose gradients, enrichment in glycosphingolipid GM1, absence of transmembrane Neural Cell Adhesion Molecule, presence of the glypiated (GPI-anchored) glycoprotein F3/F11 and of the acylated growth-associated protein GAP-43. Finally we demonstrate that part of the membrane-associated Engrailed, either expressed in COS-7 cells or endogenously present in neural tissues, is not accessible to proteolytic enzymes unless the membranes have been permeabilized with detergent. This study suggests that, in addition to their well-known presence in the nucleus, Engrailed proteins are also associated with caveolae-like vesicles that are primarily transported anterogradely into the axon, and that they can get access to a compartment compatible with secretion.

Key words: homeoprotein, Engrailed, caveolae, microdomain, neuron, cell fractionation

INTRODUCTION

Homeoproteins belong to a class of transcription factors that are characterized by the helix-turn-helix structure of their DNA-binding domain (homeodomain) (Gehring et al., 1994). These factors, first found in Drosophila and then in all metazoans, including vertebrates, regulate major developmental events (Akam, 1989; Krumlauf, 1994). They are particularly strongly expressed in the nervous system, where they participate in the establishment of the identity of several territories (Gaunt et al., 1988; Keynes and Krumlauf, 1994; Krumlauf, 1994; Rubenstein and Puelles, 1994; Bally-Cuif and Wassef, 1995), in the definition of cell lineages (Doe et al., 1988) and, probably, in later occurring events such as neurite elongation, growth cone navigation and target recognition (Miller et al., 1992; White et al., 1992; Bloch-Gallego et al., 1993).

Homeoproteins are found in the cell nucleus, as expected from their established role as transcriptional regulators. However recent reports have demonstrated that they are also present in non-nuclear cellular compartments, for example the developing axons of post-mitotic neurons (Briata et al., 1996). Furthermore, the function of these proteins has recently been extended to the regulation of protein synthesis through their specific binding to mRNAs which, indeed, occurs outside the nucleus (Dubnau and Struhl, 1996; Rivera Pomar et al., 1996).

Recently our laboratory has reported that the homeodomain of Antennapedia is internalized by cells in culture and translocated to their cytoplasm and nucleus, where it interferes specifically with the transcriptional activity of endogenous homeoproteins (Joliot et al., 1991a,b; Le Roux et al., 1993, 1995). The mechanism of translocation across the membranes is based on the physico-chemical properties of the third helix of the homeodomain (Derossi et al., 1994, 1996). The conservation of this structure among several homeodomains therefore explains why this unexpected behavior is shared by other homeodomains, shown so far for those of Hoxa-5, Engrailed and Fushi-Tarazu (Chatelin et al., 1996 and our unpublished observations).

More recently, we have also demonstrated the internalization and nuclear targeting of full-length Hoxa-5, Engrailed and

Hoxc-8 (Chatelin et al., 1996, and our unpublished results). These observations led us to propose that homeoproteins may be traded between cells (Prochiantz and Théodore, 1995). The latter hypothesis of a paracrine function has been recently illustrated in the case of Knotted-1, a maize homeoprotein that is transported with its mRNA between two abutting cells (Lucas et al., 1995). However, since plasmodesmata, the structures through which Knotted-1 passes between the cells, exist only in plants, transport of homeoproteins between animal cells would require them not only to be internalized but also secreted in spite of the absence of a signal peptide.

In this study we have used biochemical and morphological approaches to examine the intracellular localization of Engrailed-1 (En-1) and Engrailed-2 (En-2), two homeoproteins involved in the patterning of the midbrain/hindbrain territory (Bally-Cuif and Wassef, 1995; Joyner, 1996). We have focused on the possibility that these proteins are associated with a cell compartment that is compatible with secretion. We report that, in addition to their well-known nuclear localization, Engrailed proteins can be found in specific membrane fractions having the characteristics of caveolae.

MATERIALS AND METHODS

Immunoelectron microscopy

Cerebellum and mesencephalon were dissected from embryonic day 20 (E20) rats, and nerve cells were dissociated and cultured in 100 mm Petri dishes (10^7 cells/dish) for 48 hours in culture medium before fixation (Rousselet et al., 1988, 1990).

COS-7 cells transfected with the Engrailed expression plasmid alone, or co-transfected with both the Engrailed and myc-caveolin expression plasmids (see below for plasmid constructions), were cultured for 24 hours before fixation. In some experiments, live COS-7 cells transfected with Engrailed were incubated with the biotinylated cholera toxin B subunit (Sigma) diluted in culture medium (8 μg/ml) for 20 minutes, and rinsed four times (3 minutes each) in culture medium before fixation. All groups of cells were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (PB) for 2-3 hours at 4°C. They were then washed four times (5 minutes each) in 0.2 M PB containing 50 mM glycine, gently scraped and embedded in gelatin, cryoprotected, frozen, and processed for ultracryomicrotomy and immunogold labeling as described in detail elsewhere (Kleijmeer et al., 1996). Engrailed was visualized using the polyclonal Enhb-1 antibody (1/150 in PBS containing 5% fetal calf serum) for 20 minutes at room temperature (RT), and detected with Protein A-Gold 10 nm (PAG10, purchased from Dr J.W. Slot, Department of Cell Biology, Utrecht University, The Netherlands), diluted 1/30 in PBS plus 5% fetal calf serum (FCS), for 20 minutes at RT. In the double labeling experiment in which the biotinylated cholera toxin was co-detected with Engrailed, both labels were performed sequentially. First, Engrailed was labeled with PAG10 as described above; second, the sections were postfixed with 1% glutaraldehyde in PBS for 5 minutes; third, the biotinylated cholera toxin was detected using an anti-biotin antibody (Sigma, 1/10,000, 20 minutes, RT) and Protein A-Gold 5 nm (PAG5, 1/60, in PBS-5% FCS, 20 minutes, RT) as reporter. A similar sequential approach was used for the simultaneous detection of the Engrailed and myc-caveolin proteins in COS-7 cells co-transfected with the Engrailed and myc-caveolin plasmids. An anti-myc 9E10 (Evan et al., 1985) monoclonal antibody (supernatant diluted 1/50 in PBS-5% FCS, 20 minutes, RT) was used for the detection of the myc-caveolin protein. Engrailed was detected using PAG15 (or PAG10) while myc-caveolin was visualized using PAG10 (or PAG5); similar results were obtained regardless of the order of processing for protein detection (first Engrailed, second myc-caveolin or first myc-caveolin, second Engrailed), or of the size of the gold particles used (PAG15, PAG10 or PAG5). In some experiments, myc-caveolin was detected alone using PAG10 as the reporter.

Plasmid constructs

pTL1-ckEN2 expressing chicken Engrailed-2 under the SV40 promoter was made by insertion of a 1 kb *Eco*RI fragment of pSFCVLE-ckEN2 (kindly provided by S. Saule) into the *Eco*RI site of pTL1, a slightly modified version of pSG5 (Green et al., 1988). For the construction of pTL1-MycCav, the 690 bp *Eco*RI-*Xho*I fragment of pBS-VIP21 (gift of K. Simons) was inserted into pTL1 digested with the same enzymes. The *Eco*RI-*Sna*BI fragment of the caveolin insert was then removed and replaced by a 60 bp oligonucleotide containing the coding sequence of the myc tag (13 amino acids) followed by the first 15 nucleotides of the caveolin coding sequence.

Subcellular fractionation of nervous tissues

Cortex or cerebellum and posterior mesencephalon from rat E19 embryos were dissected and the meninges were carefully removed. The tissue was disrupted by 10 strokes of the Dounce homogenizer followed by 10 passages through a G26 needle in buffer H (10 mM Hepes pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, protease inhibitors) plus sucrose 0.25 M (HS0.25). The homogenate was loaded on a HS1.7 cushion (buffer H with 1.7 M sucrose) and nuclear (pellet) and membrane (interface) fractions were separated by centrifugation (100,000 ***g***, 45 minutes, 4°C) in a SW41 rotor (Beckman). Absence of nuclear or cellular contamination in the membrane fraction was checked by light microscopy.

For direct analysis the membrane fraction was analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In some cases the fraction was divided into three parts and treated as indicated in the text. Trypsin treatments (300 μg/ml, 45 minutes, 4°C) were stopped by addition of FCS (10% final concentration) and soybean trypsin inhibitor (500 μg/ml, Boehringer). Samples adjusted to 1% Triton X-100 were partially purified on heparin Ultrogel (IBF). Proteins bound to heparin Ultrogel were analyzed by 12% SDS-PAGE.

In some cases the membrane fraction was centrifuged (25 minutes, 10,000 ***g***, 4°C) in a JA-20 rotor (Beckman). Supernatants were divided into two parts, made up to 5 ml with buffer H with or without 1% Triton X-100 and centrifuged (150,000 ***g***, 45 minutes, 4°C) in a SW55 rotor (Beckman) and the pellets were analyzed by 12% SDS-PAGE.

For gradient analysis, the membrane fraction was purified on a sucrose cushion in MNB (25 mM Mes pH6.5, 150 mM NaCl), adjusted to 1% TritonX-100, 40% sucrose in 2 ml MNB and loaded at the bottom of a 10 ml linear 5%-30% sucrose gradient (in MNB). After centrifugation (200,000 ***g***, 18 hours, 4°C) in a SW41 rotor (Beckman), 1 ml fractions were collected and the proteins were precipitated with 10% trichloracetic acid (TCA) before SDS-PAGE analysis. Alternatively, whole cell homogenates (in 1% Triton X-100, 40% sucrose, 2 ml MNB) were loaded at the bottom of the gradient. When specified, the homogenate was incubated with 0.2% saponin before loading at the bottom of the gradient. 1 ml fractions were analyzed after TCA precipitation, or subdivided. In the latter case, half of the fraction was TCA precipitated, the other half was diluted tenfold in MNB buffer in the presence of 1% Triton and centrifuged (150,000 ***g***, 45 minutes) in a SW 55 rotor (Beckman). The supernatant was precipitated with TCA, and the pellets were run on 7%-18% polyacrylamide gels.

Gradient analysis of transfected COS-7 cells

COS-7 cells (5×10^6) were electroporated (170 V, 950 μF with Biorad gene pulser II) with pTL1-ckEN2 (65 μg) with or without pTL1-MycCav (20 μg). 24 hours later, the cells were washed twice with MNBS (MNB, 0.25 M sucrose) and scraped in 2×1 ml MNBS containing 1% Triton X-100 and protease inhibitors. Cell extracts were adjusted to 40% sucrose and 1% Triton X-100 in 2 ml MNB with or

without 0.2% saponin. After a 30 minute incubation with gentle agitation, cell extracts were centrifuged (15,000 ***g***, 5 minutes, 4°C) and the supernatants were loaded at the bottom of a 10 ml linear 5%-30% sucrose gradient (in MNB). After centrifugation (200,000 ***g***, 18 hours, 4°C) in a SW41 rotor, 1 ml fractions were collected and the proteins were precipitated with TCA before SDS-PAGE analysis.

Western blotting

Proteins were transfered on nitrocellulose (BAS 85, Schleicher & Schuell). Rat Engrailed and chick-Engrailed proteins were detected with the polyclonal Enhb-1 (1/10,000) and the monoclonal 4D9 (1/100) (Patel et al., 1989) antibodies, respectively. Myc-caveolin was revealed with the 9E10 monoclonal antibody (1/20). The anti-NCAM (1/1000) and anti-F3 (1/200) polyclonal antibodies were generous gifts of C. Goridis and G. Rougon, respectively. The anti-RNA polymerase II monoclonal antibody (1/5000) was obtained from O. Bensaude. Primary antibodies were detected with the ABC peroxidase kit (Vector) followed by ECL (Amersham). Antibody incubations and washes were performed in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.20% Tween 20) with 5% non-fat dried milk. Streptavidin peroxidase incubation and following washes were performed in 2× TBS, 0.20% Tween 20.

RESULTS

The polyclonal Enhb-1 antibody directed against mammalian Engrailed-1 (En-1) and Engrailed-2 (En-2) was used to detect these proteins on tissue sections from cerebellum, posterior tectum and cortex from post-natal day 1 (PN1) mice. As expected, the staining was specific for the tectum and cerebellum, and no signal was observed in the cortex (not shown). To characterize the precise localization of Engrailed proteins at the electron microscopic level, we used immunogold labeling on ultrathin frozen sections of paraformaldehyde-fixed cells, allowing both preservation of ultrastructure and high sensitivity of labeling. Unfortunately, only 10-20 gold particles in individual nuclear profiles (50 nm sections) could be observed. This low endogenous labeling was clearly not sufficient to characterize further the neuronal compartmentation of Engrailed proteins, despite the indication by cell fractionation studies that non-nuclear Engrailed was present (see below).

To overcome this difficulty we first transfected COS-7 cells with chick-Engrailed-2 (c-En). Fig. 1, documenting the ultrastructural localization of c-En, shows that this protein is present in the nucleus and cytoplasm of transfected COS-7 cells. The background was extremely low; only occasional gold particules were observed in non-expressing compared to expressing cells (Fig. 2). The number of gold particles in the nuclei was very variable from one transfected cell to another and did not seem to correlate with the c-En extranuclear concentration (for example, compare Figs 1A and 3A). In contrast to neurons, the low nucleocytoplasmic ratio of these cells allows the observation of a large number of gold particles in the cytoplasm, where

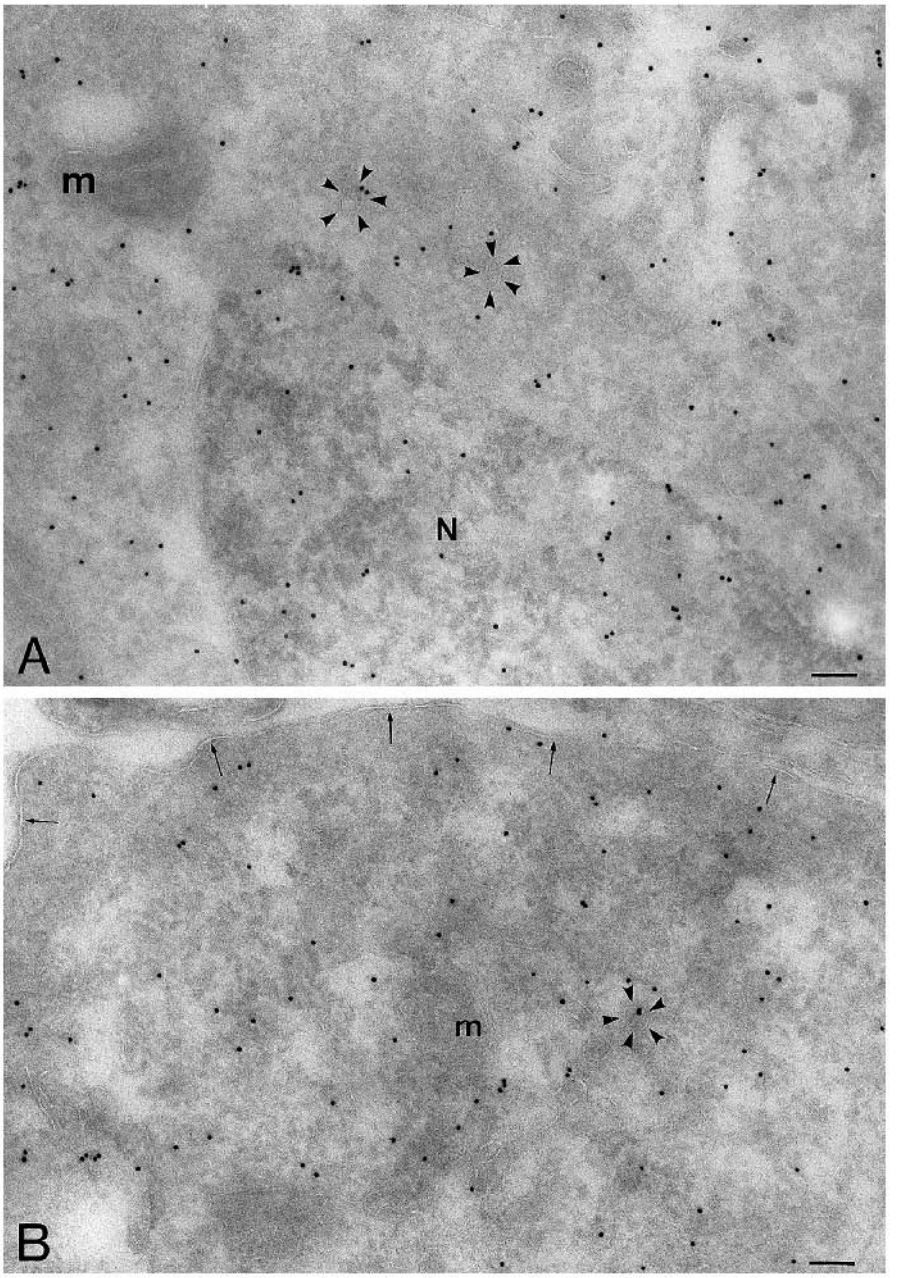

Fig. 1. Subcellular localization of chick Engrailed-2 in transfected COS-7 cells; two examples showing nuclear (A) and cytoplasmic (A,B) compartments. Ultrathin cryosections were immunogold-labeled for Engrailed (PAG10). Gold particles are observed in the nucleus (N), as well as in the cytoplasm. While many gold particles are observed within the cytosol, a significant amount of label is found in the vicinity of caveolae-like vesicles (examples of such vesicles are indicated with arrowheads in A and B) and close to the plasma membrane (thin arrows in B). By contrast, no significant label is observed in association with the external membrane of mitochondria (m). Bars, 100 nm.

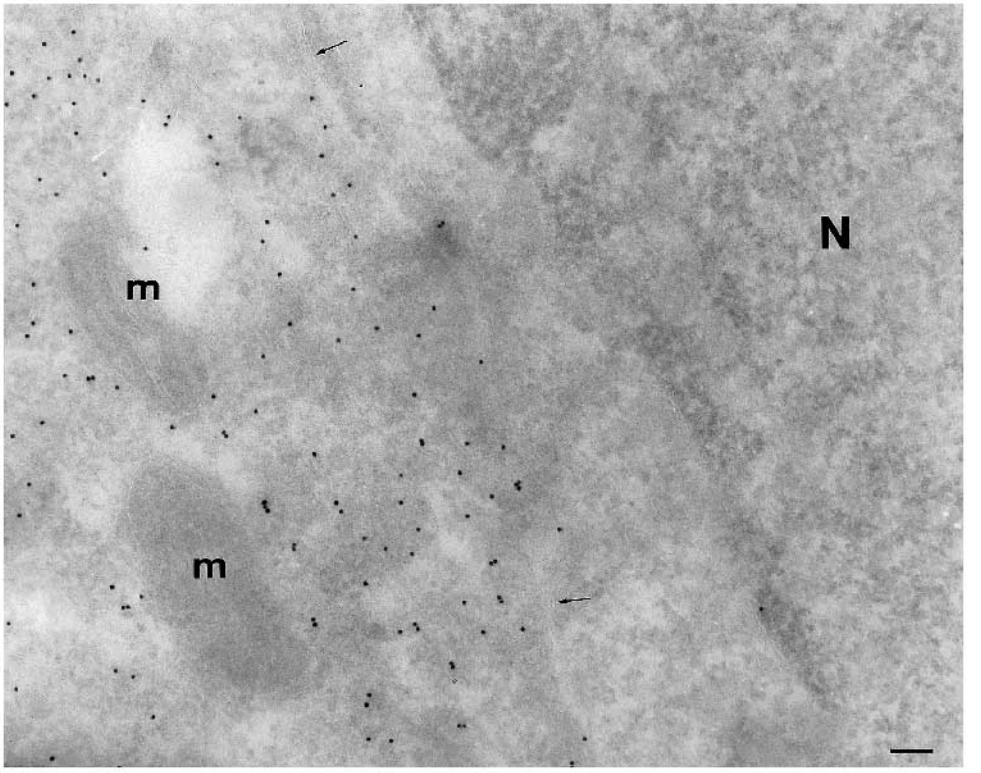

Fig. 2. Immunogold detection of c-En is highly specific. Two neighbouring COS-7 cells, expressing (left) or non-expressing (right) Engrailed are shown. The cryosection was labeled for Engrailed (PAG10). This picture demonstrates the high signal to noise ratio of the staining, as well as the very low background: only occasional gold particules are observed in the cytoplasm or nucleus (N) of the non-expressing cell. In the expressing cell, no label is found in the mitochondria (m). Arrows indicate the plasma membrane of the non-expressing cell. Bar, 100 nm.

they are often found in the close vicinity of membranes and, in particular, of flask-shaped, non-coated vesicles that are morphologically similar to caveolae, with a diameter ranging from 50 to 100 nm (Fig. 1).

Caveolae are non-coated membranous structures that can be identified by the presence of caveolin, a 21 kDa protein, and of specific glycosphingolipids such as GM1, the cholera toxin receptor. To further characterize the membrane vesicles with which c-En co-localizes, we incubated c-En-transfected COS-7 cells for 20 minutes with biotinylated cholera toxin B subunit, which binds GM1, and revealed both GM1 and Engrailed by double-immunogold labeling on ultrathin frozen sections. As illustrated in Fig. 3, c-En is often found associated with membrane vesicles containing internalized cholera toxin (see arrowheads in Fig. 3). Furthermore, a significant double staining is observed at the surface of the cells, on the cytoplasmic side of the plasma membranes (Fig. 3A).

Transfecting cells with caveolin has been shown to increase

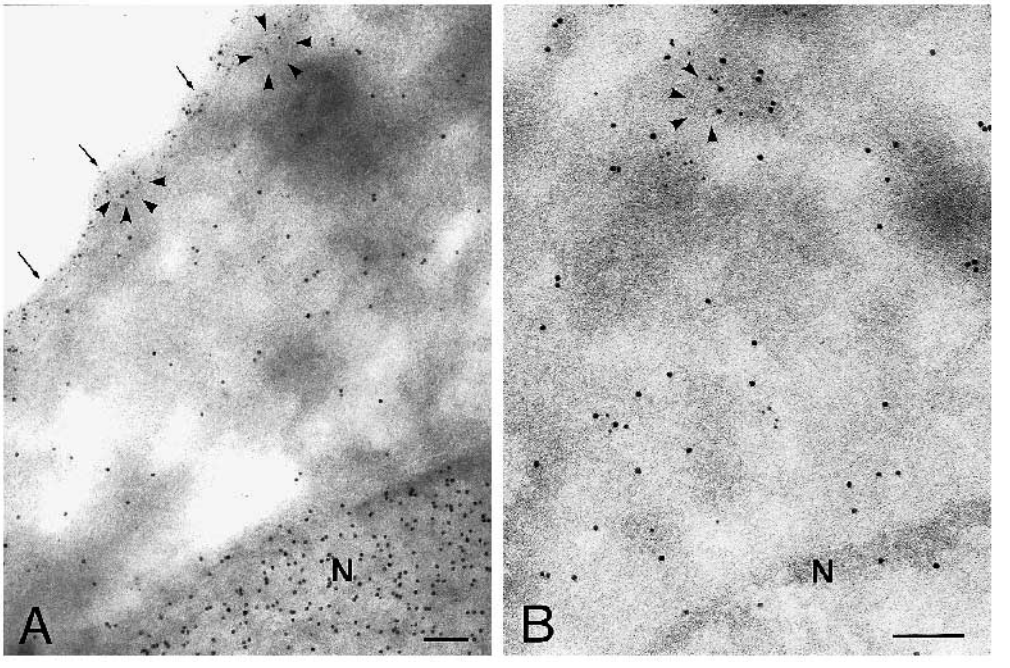

Fig. 3. Colocalization of chick Engrailed-2 with cholera toxin. COS-7 cells transfected with the Engrailed-2 expression vector were incubated with biotinylated cholera toxin B subunit for 20 min at 37°C and rinsed several times in culture medium before fixation. Cryosections were immunogold-labeled for both Engrailed-2 (PAG10) and cholera toxin (PAG5). Cholera toxin labeling is abundant at the plasma membrane (thin arrows), and is observed in vesicles mainly located in the peripheral cytoplasm (A). Gold particles detecting Engrailed-2 are observed in the nucleus (N) and in the cytoplasm. Both Engrailed and cholera toxin colocalize in plasma membrane domains (A, thin arrows) and within individual caveolae-like vesicles (A,B, arrowheads). Bars, 100 nm.

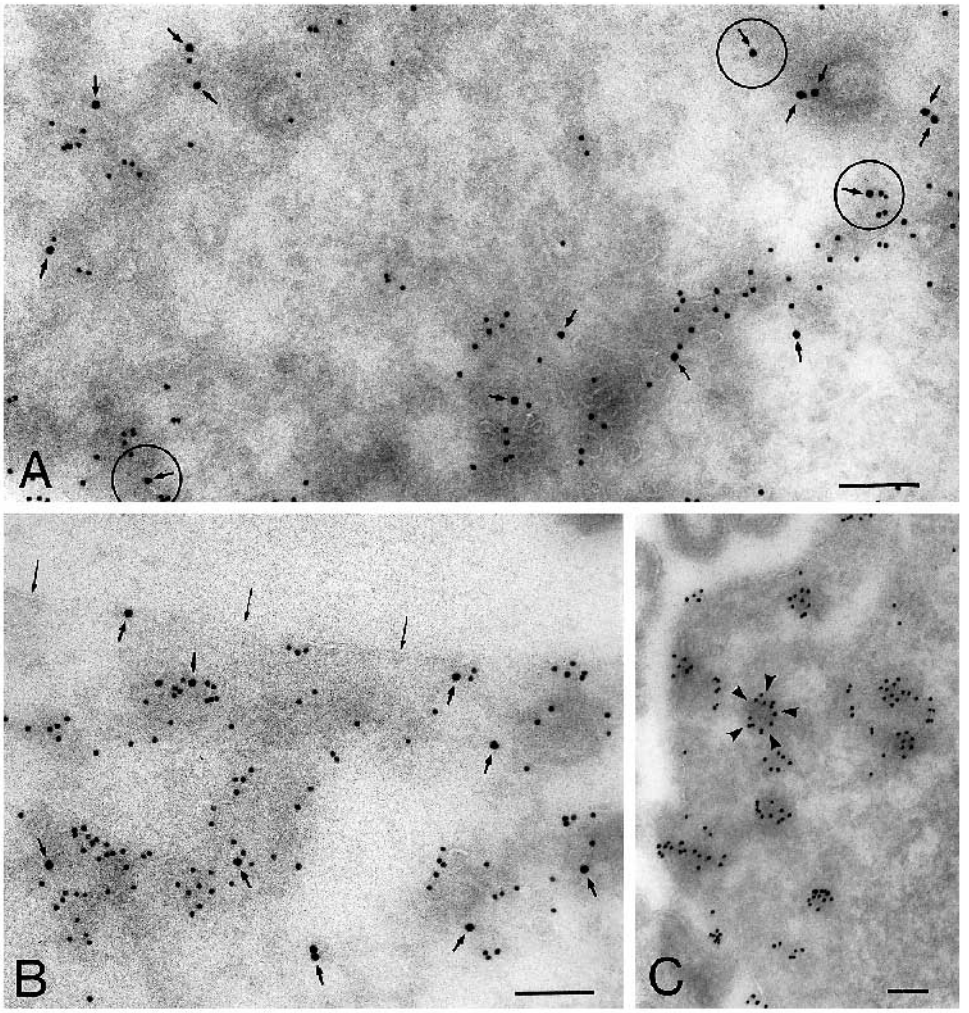

Fig. 4. Colocalization of chick Engrailed-2 and myc-caveolin in doubly transfected COS-7 cells. Cryosections from COS-7 cells co-transfected with the Engrailed and the myc-caveolin expression plasmids were labeled for myc-caveolin (PAG10) alone (C) or double-labeled for Engrailed (PAG15) and myc-caveolin (PAG10, A,B). In A and B protein A binding sites were deactivated with glutaraldehyde after Engrailed staining (see Methods), which decreases the reactivity of the myc epitope, so that the decoration of caveolae with PAG10 beads is less intense than in C (arrowheads). A majority of Engrailed gold particles (short arrows) is found in the vicinity of myc-caveolin beads (the diameter of circles around three Engrailed beads that were taken as examples is 90 nm) or in direct contact with caveolae-like vesicles expressing myc-caveolin. Some staining is in proximity of the plasma membrane (thin arrows in B) and large regions of the cytoplasm, devoid of caveolae and myc staining, show only a few gold particles detecting Engrailed. Bars, 100 nm.

the number of caveolae in lymphocytes (Fra et al., 1995). Therefore we co-transfected COS-7 cells with c-En expression plasmid and with a construct coding for a myc-tagged caveolin protein (myc-caveolin), allowing its detection with a specific anti-myc monoclonal antibody (9E10). As demonstrated by Kurzchalia et al. (1992), the presence of the myc epitope does not hamper the association of the protein with caveolae. Fig. 4 shows that transfection with caveolin increases the number of caveolae (Fig. 4C), and that c-En (arrows in Fig. 4A,B) and caveolin are enriched in the same general domains of the cell, in the vicinity of caveolar structures. The amount of c-En present in the cytoplasm and associated with membranes bearing (or not bearing) caveolin has been quantified. Table 1 indicates that 3-13.5% (mean: 7.6%) of non-nuclear c-En is closely associated with caveolae (within 15 nm of the membrane). The remaining gold particles detecting c-En were either associated with myc-negative non-coated (caveolae-like) vesicles (mean: 3.5%), free in the cytoplasm, or associated with ill-preserved membranes.

Due to their high glycosphingolipid and cholesterol content, caveolae are resistant to Triton X-100 and are light on sucrose density gradients (Parton, 1996). They can thus be isolated and identified by the presence of caveolin. Such fractionations, first done with COS-7 cells over-expressing c-En, show a significant amount of c-En in the high density fractions (Fig. 5, top, fractions 1-3), which contain the bulk of membranes and the soluble molecules, including the nuclear proteins. In addition, c-En is present in light density fractions 5-8, even though there are no proteins detectable by Coomassie blue staining in these fractions (not shown).

Co-transfection with myc-caveolin demonstrates that both c-En and myc-caveolin can be detected in the same fractions, in particular fractions 4-6 (Fig. 5, middle). The modification in c-En distribution observed when myc-caveolin is over-expressed is probably due to the density shift of the membranes provoked by their higher content of transfected caveolin (well illustrated in Fig. 4C); this strongly suggests that c-En and caveolin are associated with the same sub-population of membranes.

Saponin, a cholesterol-binding detergent, is known to disrupt caveolae (Rijnboutt et al., 1996). The Triton extracts were thus incubated with saponin and run on a sucrose gradient. As shown in Fig. 5 (bottom), the addition of saponin solubilizes caveolae-associated myc-caveolin, which now runs with the soluble molecules. The same mobility shift is observed for c-En, suggesting once more a co-association of caveolin and c-En with cholesterol-enriched membranes. The marked decrease in c-En and myc-caveolin signals in fraction 4 after saponin treatment indicates that both proteins can also be associated with cholesterol-enriched membranes of a relatively high density. This observation reinforces the hypothesis that

Table 1. Semi-quantitative analysis of cytoplasmic immunogold labeling in COS-7 cells co-transfected with c-En and myc-caveolin

	Cell	Free PAG10 (%)	PAG10/PAG5 (%)	PAG10/vesicle (%)
Grid 1	1	**235** (75)	**42** (13.5)	**36** (11.5)
	2	**141** (84.5)	**15** (9)	**11** (6.5)
	3	**97**(88.3)	**3** (2.7)	**10** (9)
	4	**218** (87)	**24** (9.5)	**8** (3.5)
	5	**243** (93.5)	**8** (3)	**9** (3.5)
	6	**49** (91)	**2** (3.5)	**3** (5.5)
	7	**662** (88.5)	**74** (10)	**11** (1.5)
	8	**812** (92)	**61** (7)	**10** (1)
	9	**262** (92)	**15** (5)	**8** (3)
	10	**515** (87.5)	**53** (9)	**20** (3.5)
Grid 2	11	**59** (89)	**7** (10.6)	**0** (0)
	12	**107** (95)	**4** (3.5)	**2** (1.7)
	13	**510** (85.7)	**68** (11.4)	**17** (2.9)
	14	**383** (92)	**28** (6.8)	**5** (1.2)
	15	**96** (88.8)	**7** (6.5)	**5** (4.6)
	16	**379** (92.4)	**22** (5.4)	**9** (2.2)
	17	**155** (89.1)	**13** (7.5)	**6** (3.4)
	18	**403** (89.6)	**33** (7.3)	**14** (3.1)
	19	**514** (87.6)	**61** (10.4)	**11** (2)
	20	**348** (92.3)	**27** (7.2)	**2** (0.5)
	21	**319** (86.9)	**38** (10.4)	**10** (2.7)

Engrailed was detected with PAG10 and myc-caveolin with PAG5. 21 individual co-transfected cells displaying a significant cytoplasmic c-En staining were analyzed on two different grids (Grid 1 and Grid 2). The cytoplasmic distribution of c-En was estimated as follows: Free PAG10: PAG10 gold particles observed in the cytosol, with no apparent direct association with either PAG5 or vesicles (cytosolic c-En). PAG10/PAG5: PAG10 gold particles associated with PAG5 particle-labeled vesicles (c-En associated with caveolae). In this calculation we only counted PAG10 particles that were directly associated with a membrane decorated with a PAG5 particle (distance between particles less than 15 nm). With reference to Fig. 4A,B, this has led us to ignore all Engrailed particles in the vicinity of a myc-caveolin bead (e.g. within a 100 diameter circle) but at a greater distance than 15 nm. PAG10/vesicle: PAG10 particles associated with vesicles not labeled by PAG5 (c-En associated with caveolae-like vesicles that were apparently devoid of caveolin).

over-expression of myc-caveolin induces caveolae formation of unusual density without detectable alterations at the ultrastructural level.

Nervous tissues do not contain any of the three caveolin proteins identified so far (Scherer et al., 1996; Song et al., 1996), implying that, stricto-sensu, caveolae are not present in neurons. However, neurons contain membrane microdomains, in particular subplasmalemmal vesicles, with biochemical traits characteristic of classical caveolae. Among the latter, high cholesterol and glycosphingolipid content are responsible for the Triton resistance and low density on sucrose gradients of both caveolae and microdomains (Bouillot et al., 1996).

In a first experiment we verified whether embryonic day 19 rat En-1 and En-2 could be recovered in a membrane fraction floating on a 1.7 M sucrose step and then concentrated at 150,000 ***g***. Fig. 6 (left) illustrates that a significant amount of Engrailed protein is associated with the membrane pellet, which is not solubilized in the presence of Triton X-100 (Fig. 6, right). En-1 and En-2 were dissociated from the membranes with 0.5 M NaCl (Fig. 6, middle), suggesting that they are associated with the membranes, through electrostatic interactions.

We then subjected embryonic neurons to the fractionation technique in which Triton-resistant membranes are separated on a sucrose density gradient. The low density fractions are enriched in microdomains, as demonstrated in a previous study (Bouillot et al., 1996). Since the distributions of En-1 and En-2 were identical, only En-2, which is more strongly expressed at this stage of development, is shown. As seen in Fig. 7A, En-2 is present in fractions 1-4 where the soluble molecules and the bulk of membranes are present, but also in lighter fractions, in particular fractions 6 and 7. To verify the purity of the light fractions we used both positive and negative markers. Fig. 7A shows that fractions 4-10 are totally devoid of the transmembrane and polysialylated neuronal adhesion molecules NCAM140 and 180, which are present in fractions 1-3 (GPI-anchored NCAM120 is not

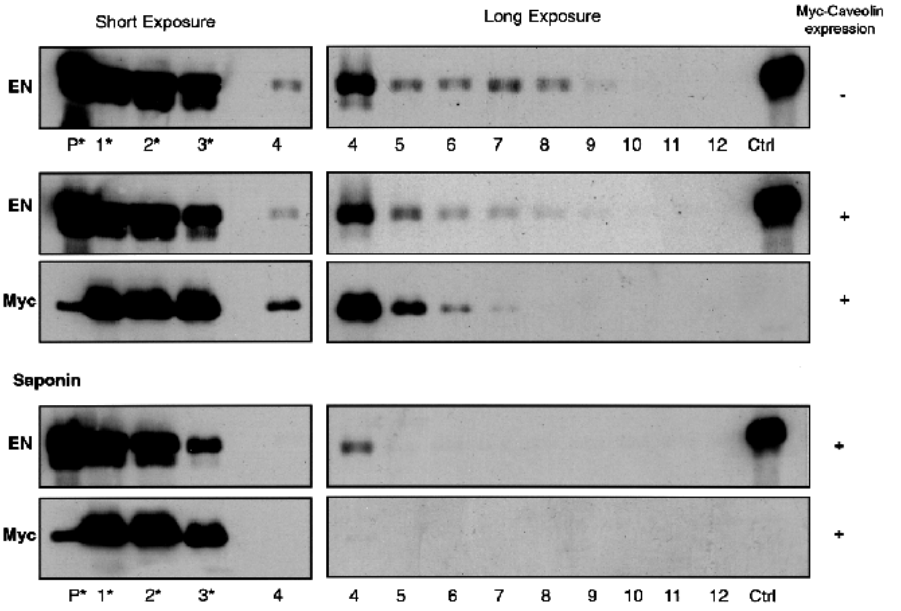

Fig. 5. COS-7 cells fractionation. COS-7 cells were electroporated with c-En expression plasmid either alone or with myc-caveolin expression plasmid. 24 hours after electroporation, cells were disrupted in MBS 1% Triton with or without 0.2% Saponin. Homogenates were separated on sucrose density gradients and each 1 ml fraction was analyzed by 12% SDS-PAGE (P, pellet; 1-12, bottom to top fractions). The blots were exposed for different times (short or long exposure). For comparison, fraction 4 is shown at both exposures. In the absence of myc-caveolin, significant amounts of Engrailed migrate in the light fractions 5-9 (top). Transfection with myc-caveolin (middle) slightly shifts Engrailed distribution toward higher density fractions. Adding saponin, a cholesterol-binding detergent (bottom), solubilizes both Engrailed and caveolin, which are no longer found in fractions 5 to 9. En, c-Engrailed-2; myc, exogenous myc-caveolin; Ctrl, nuclear extract of c-En transfected cells run on the same gel as a positive control for Engrailed immunoreactivity. *Because of their high protein content only 1/3 of the total fraction volume was loaded.

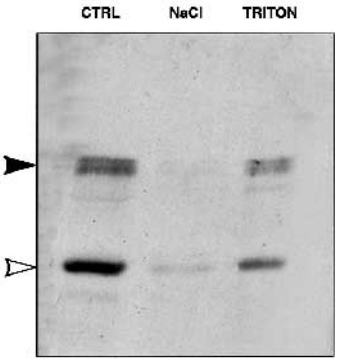

Fig. 6. En-1 and En-2 from rat neural tissues are associated with Triton-resistant membranes. Tectal and cerebellar membranes floated on 1.7 M sucrose were cleared by centrifugation (10,000 ***g***; 25 minutes) and pelleted (150,000 ***g***; 45 minutes) in the absence (Ctrl) or presence of either 0.1% Triton X-100 (Triton) or 0.5 M NaCl (NaCl). En-1 (black arrowhead) and En-2 (white arrowhead) are found in the high speed pellet. Association with the membranes is lost at high salt but partially preserved in the presence of Triton X-100.

expressed at this stage). Fig. 7A also shows the distributions of F3/F11 GPI-anchored glycoprotein and of GAP-43. F3/F11 is present in fractions 6 and 7, but also in even lighter fractions 9 and 10. As for GAP-43, an acylated protein enriched in the axonal shaft and growth cone and associated with subplasmalemmal vesicles, its distribution is identical to that of En-2. Because the Triton treatment and separation on sucrose gradient were performed with a purified membrane fraction, it is unlikely that residual nuclear contamination is responsible for the presence of Engrailed in light density fractions. To further eliminate this possibility, we analyzed the distribution of the nuclear-specific marker RNA polymerase II (Krämer et al., 1980). In this experiment, in order to enrich the Triton X-100 material with nuclear components, whole cell homogenates from cerebellum or posterior tectum, and not purified membranes, were loaded at the bottom of the gradient. As demonstrated in Fig. 7B, RNA polymerase II is absent from the light density fractions obtained from tectum homogenate, which nevertheless contain significant amounts of Engrailed. We also observed a significant and reproducible difference between cerebellum and posterior tectum, with a higher amount of Engrailed in fractions 6 and 7 from the latter structure (Fig. 7B). Because this difference does not simply reflect variations of the total amount of Engrailed protein in the cell homogenates (Engrailed signal in high density fractions is comparable in both structures), we have initiated a quantitative analysis of the intracellular distribution of Engrailed during ontogeny, which will be part of another study.

In the fractionation experiments shown in Fig. 7, the total protein content of each fraction was determined by TCA precipitation before analysis. This procedure did not allow us to ascertain, in each fraction, how much Engrailed protein is actually associated to membranes. To investigate this point, half of each fraction was precipitated with TCA and the other half was centrifuged at 150,000 ***g*** to evaluate the amount of soluble (supernatant) versus membrane-associated (pellet) Engrailed. Fig. 8 illustrates that in the light density fractions (e.g. fraction 7, representative of fractions 5-7) all Engrailed is associated with the microdomains, whereas in the high density fractions (e.g. fraction 2, representative of fractions 1-3) the protein is almost entirely soluble. Furthermore, when microdomains are solubilized by the cholesterol-binding detergent saponin, Engrailed is no longer present in the light density fractions (right panel).

Finally, we investigated whether some of the Engrailed molecules present in the membrane fraction could get access to a compartment where they would be protected against proteolytic degradation. To this end the membrane fraction floating on 1.7 M sucrose was treated with proteinase K or

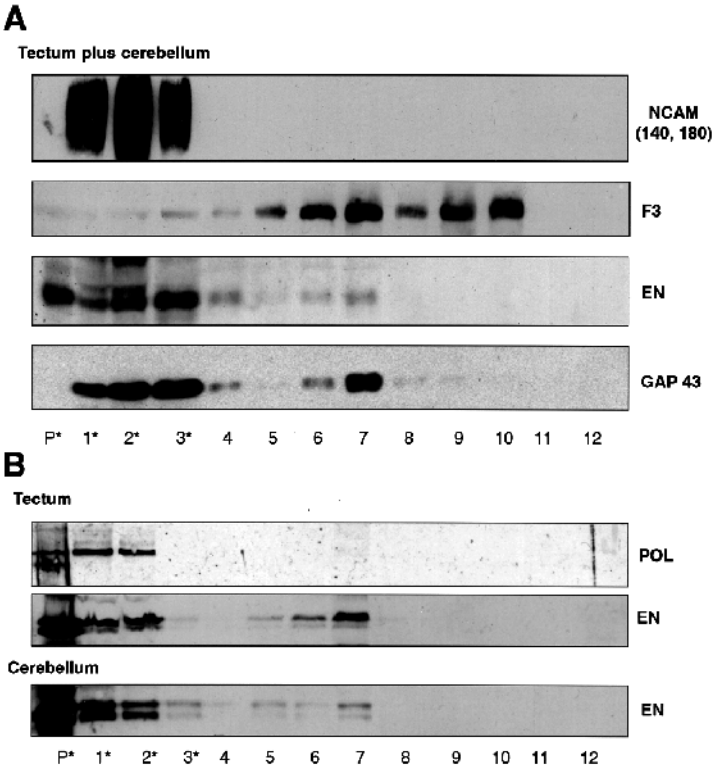

Fig. 7. Engrailed from neural tissues is present in Triton-resistant and low density membrane fractions. Membrane fractions (A) from rat E19 cerebellum and tectum or whole cell homogenates from either cerebellum or tectum (B) were treated with 1% Triton X-100 and separated on a sucrose density gradient. Pellet (P) and 1 ml fractions (1-12, bottom to top) were loaded on 7% to 18% gradient (A, F3 and NCAM; B) or 10% SDS-polyacrylamide gels (A, EN and GAP 43).
(A) Transmembrane NCAM 140 and 180 (top) accumulate only in the heavy density fractions 1-3, just above the pellet (P). GPI-anchored glycoprotein F3/F11 (F3) is not present in the heavy fractions and is recovered from fractions 5-10. Engrailed is found both in the heavy fractions, including the pellet, where most soluble molecules are present at equilibrium, and in light fractions 6 and 7 (EN), like GAP-43, an acylated molecule anchored in subplasmalemmal axonal vesicles (bottom).
(B) RNA polymerase II (POL) from tectum, used as nuclear marker, is detected only in the pellet and in fractions 1-2. En is present in both heavy and light fractions from both cerebellum and tectum. *Because of their high protein content only 1/3 of the total volume of these fractions was loaded.

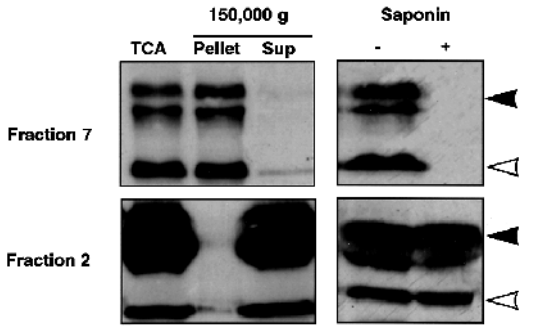

Fig. 8. Soluble and membrane-associated Engrailed in light and heavy density fractions. Whole cell homogenates from posterior tectum were analyzed as in Fig. 7B, in the presence or absence of saponin. (Left) The total content of Engrailed protein in light density fraction 7, as estimated by TCA precipitation (TCA), is fully recovered in Triton-insoluble membranes (Pellet) after 150,000 ***g*** centrifugation, whereas Engrailed localized in high density fraction 2 remains in the supernatant (Sup). (Right) Saponin treatment of the cell homogenates before sucrose gradient separation totally abolishes the migration of Engrailed protein in fraction 7 without having any effect on high density fractions. Black arrowhead, Engrailed-1; white arrowhead, Engrailed-2.

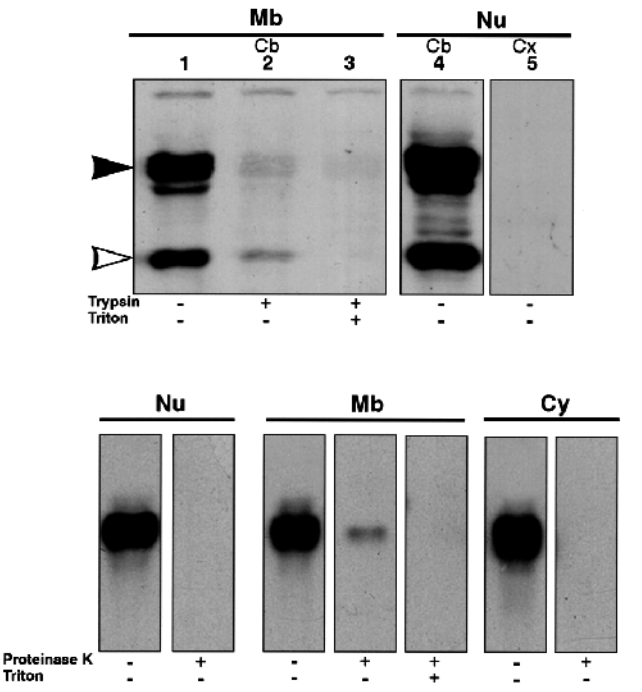

Fig. 9. Resistance to proteolysis of membrane-associated Engrailed. (Upper panel) Equivalent amounts of membrane fraction from cerebellum (Cb) floating on 1.7 M sucrose were either non-treated or treated with trypsin (300 μg/ml) in the presence or absence of 0.1% Triton X-100. After addition of trypsin inhibitors, the samples were partially purified on Heparine-Ultrogel and analyzed by 10% SDS-PAGE. As seen in lane 2, small amounts of En-1 (black arrowhead) and En-2 (white arrowhead) are resistant to proteolysis unless the membranes are permeabilized (lane 3). Lanes 4 and 5 illustrate the high specificity of the antibody with nuclear fractions from cerebellum (Cb) and cortex (Cx). (Lower panel) Membrane, cytosolic and nuclear fractions of COS cells transfected with the c-En-expressing plasmid were either non-treated or treated with proteinase K (100 μg/ml) in the presence or absence of 0.1% Triton-X100. After TCA precipitation, samples were analyzed by 12% SDS-PAGE. Protection against degradation is only seen in the membrane fraction and is lost upon permeabilization. Mb, membranes; Nu, nucleus; Cy, cytosol; Cb, cerebellum; Cx, cortex.

trypsin before high speed centrifugation and analysis. Fig. 9 (top panel) illustrates that membranes from the cerebellum contain a small but significant amount of Engrailed, which is protected against proteolysis, and that this protection is lost after permeabilization of the membranes by the addition of Triton. Similar results were obtained with c-En transfected COS-7 cells (Fig. 9, bottom panel); this figure further shows that c-En present in the soluble (cytosolic) or nuclear fractions is not protected against proteolysis.

DISCUSSION

Caveolae are vesicles for which a function in signal transduction has been proposed (reviewed in Parton, 1996). Often situated at the apical domain of polarized cells, they are characterized by the absence of a clathrin coat, the presence on their cytoplasmic side of a 21 kDa protein named caveolin, and an enrichment in glycosphingolipids (e.g. GM1) and cholesterol. GPI-anchored glycoproteins are found associated with caveolae, or become associated with them during extraction in the presence of Triton X-100. Their high cholesterol content confers two highly specific properties to caveolae: Triton X-100 resistance and low density on sucrose gradients. Membrane structures enriched in GM1, GPI-anchored proteins and cholesterol can form in the absence of caveolin (Gorodinsky and Harris, 1995). Like caveolae, the latter structures are resistant to Triton X-100 and of low density in sucrose gradients. They are present in neurons, where none of the three known caveolins (Parton, 1996; Scherer et al., 1996; Song et al., 1996) has been found, and have been called caveolae-like or microdomains (Gorodinsky and Harris, 1995; Bouillot et al., 1996). We report here that a significant fraction of the transcription factor Engrailed is associated with these microdomains, both in vivo and in vitro.

Specificity of the association of Engrailed with microdomains and caveolae

There are several arguments in favor of a specific interaction between Engrailed and microdomains with the characteristics of caveolae.

Firstly, as illustrated in Figs 1 and 3, the presence of Engrailed in the vicinity of non-coated vesicles and of smooth membranes does not directly correlate with the amount of protein in the nucleus, strongly suggesting that such an association is not due to over-expression in transfected cells. Observations at the ultrastructural level also demonstrate that the distribution of Engrailed is not homogeneous within the cytoplasm. For example, no staining is ever seen within or at the surface of mitochondria, and Engrailed is preferentially present in regions where cholera toxin, a specific marker of GM1 glycosphingolipid, accumulates after uptake by live cells (Fig. 3). The bias towards an association with myc-caveolin, which has been shown to enhance the number of caveolae (Fra

et al., 1995), also suggests a specific interaction of Engrailed with these membrane domains.

Secondly, Triton-resistance and low density on sucrose gradients are highly specific properties of microdomains and caveolae. The validity of the fractionation protocol used in this study has been verified, including at the ultrastructural level (Bouillot et al., 1996). The fractions prepared from rodent cerebellum and tectum consist of true microdomains, as demonstrated by their GM1 content (not shown), the presence of GPI-anchored glycoprotein F3/F11 (Gennarini et al., 1989; Faivre-Sarrailh et al., 1992), the absence of transmembrane NCAM (Fig. 7A) and of nuclear protein RNA polymerase II (Fig. 7B). The specific association of Engrailed proteins with microdomains is further confirmed by the observation that the presence of the homeoprotein in light density fractions depends on its association with cholesterol-enriched (solubilized by the cholesterol-binding detergent saponin) and Triton-insoluble membranes (Fig. 8), two major properties of microdomains.

In the case of COS-7 cells, c-En is found in light density fractions that also contain caveolin when this protein is overexpressed. In these fractions, both Engrailed and caveolin are solubilized by the addition of saponin, which disrupts the caveolae (Rijnboutt et al., 1996). This solubilization results in the redistribution of both caveolin and Engrailed toward higher density fractions (Fig. 5). In addition, the finding that overexpression of myc-caveolin induces a mobility shift of c-En on sucrose gradients is further evidence that both proteins are associated with the same vesicules.

Finally, in the light fractions (fractions 6-10 in Fig. 7A), which are enriched in F3/F11, Engrailed is primarily present in fractions 6 and 7, suggesting a more specific association with a particular subfraction of the microdomains and certainly precluding a random association with the membranes during tissue homogeneization. It is noteworthy that fractions 6 and 7 also contain the GAP-43 protein, which is anchored to non-coated vesicles in the growth cones of elongating axons (Benowitz and Routtenberg, 1987; Skene and Virag, 1989; Bedge et al., 1992). Because F3/F11 is present in intracellular vesicles and at the cell surface, the experiment of Fig. 7A suggests that fractions 6 and 7 are enriched in intracellular vesicles (where GAP-43, Engrailed and some F3/F11 would be present) whereas fractions 8-10 are enriched in microdomains from the plasma membrane (in which only F3/F11 would be incorporated).

Intracellular compartmentalization of homeoproteins

The expression patterns of homeoprotein messenger RNAs and the genetic analysis of homeogenes have been the focus of a very large number of scientific reports. By contrast, probably because of the lack of specific antibodies, interest in the tissue and cellular distribution of homeoproteins is very recent. It has, however, already led to rather unexpected observations, in the light of which the present results will now be discussed.

Firstly, homeoproteins Emx-1 and Hoxa-7 have been found in the axon and nerve terminals of post-mitotic neurons. Emx-1, a homeoprotein expressed in the developing forebrain, is found in the axon of the mouse olfactory receptors (Briata et al., 1996). The staining is intense and clearly visible along the entire axon as well as at the level of the olfactory bulb glomeruli, probably, and according to the authors, within the axon terminals.

The presence of Hoxa-7 in axons has also been observed by Cory Abate and colleagues, who have established that Hoxa-7 is present in the ascending and descending spinal tracts and can therefore be found in the brain, where no Hoxa-7 transcripts are present. Using a cell culture analysis, they have also observed that Hoxa-7 is primarily located in the axon as well as in the growth cones. The latter point is substantiated by a cell fractionation study showing a co-fractionation of GAP-43 and Hoxa-7, which is very reminiscent of our own results (Cory Abate-Shen, personal communication).

The present study showing that Engrailed specifically associates with neuronal microdomains suggests a mechanism for the axonal transport of some homeoproteins. Indeed, microdomains and GPI-anchored molecules are primarily, although not exclusively, located to the axon, which has been proposed to be the neuronal equivalent of the apical domain of epithelial cells (Dotti and Simons, 1990; Dotti et al., 1991). Axonal localization of homeoproteins has so far only been observed for Emx-1 and Hoxa-7, possibly because such axonal localization is exceptional. Other explanations are the lack of good antibodies, the small number of studies centered on the cell biology of homeoproteins, particularly in post-mitotic neurons, and the low levels of expression at the stages when most immunostainings have been achieved. Indeed, the cell fractionation experiments presented in this study clearly indicate that it is only a small fraction of Engrailed that associates with the microdomains.

Secondly, very surprising findings in the recent literature are the RNA-binding properties of bicoid and the new role for this transcription factor as a regulator of mRNA translation (Dubnau and Struhl, 1996; Rivera Pomar et al., 1996). This observation demonstrates that homeoproteins can have functions requiring their presence outside the nucleus. The correlation between the axonal transport and mRNA-binding properties of some homeoproteins suggests that they might serve to locate RNAs into specific intracellular domains. Although it is quite difficult to understand the reason for their presence in a compartment that is generally thought to be devoid of ribosomes, it cannot be ignored in this context that mRNAs have been found in several axons, including those of the olfactory receptors (Mohr and Richter, 1992; Tiedge et al., 1993; Trembleau et al., 1994; Vassar et al., 1994).

Is there any link between the association of Engrailed with microdomains and the translocation of homeoproteins across biological membranes?

In 1991, we demonstrated that the homeodomain of Antennapedia (Antp-HD) was internalized by live cells in culture and addressed to their cytoplasm and nucleus where it can specifically regulate transcription (Joliot et al., 1991a,b; Bloch-Gallego et al., 1993; Le Roux et al., 1993, 1995). This property was later extended to other homeodomains, including that of Engrailed (G. Mainguy and A. Prochiantz, unpublished results), and to full-length homeoproteins (Chatelin et al., 1996). In the case of Antp-HD, translocation is due to the structure of its third helix and does not require classical endocytosis since it happens at 4°C (Derossi et al., 1994). The internalization of the reverse helix and of a helix entirely composed of D-amino acids suggests that no chiral receptor is required for internalization (Derossi et al., 1996).

Based on these observations, it was proposed that translocation requires both cholesterol, because of the low permissive

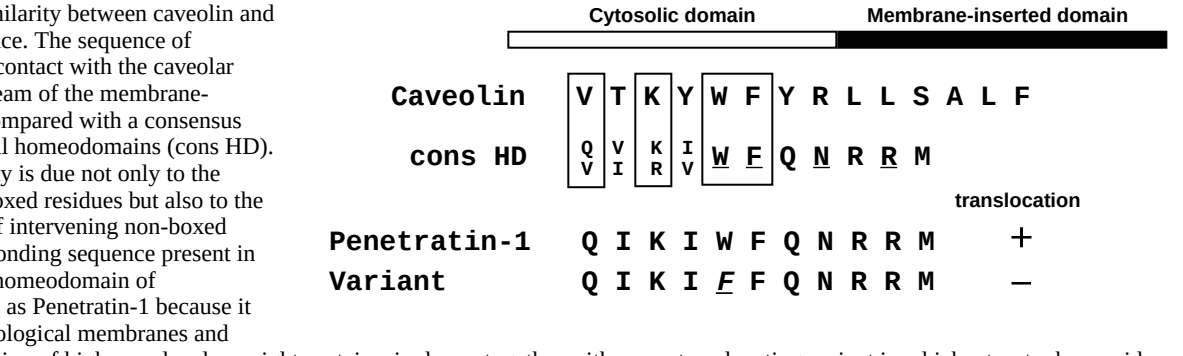

Fig. 10. Sequence similarity between caveolin and HD consensus sequence. The sequence of caveolin, which is in contact with the caveolar membrane, just upstream of the membrane-inserted domain, is compared with a consensus sequence present in all homeodomains (cons HD). Note that the similarity is due not only to the conservation of the boxed residues but also to the hydrophobic nature of intervening non-boxed residues. The corresponding sequence present in the third helix of the homeodomain of Antennapedia, known as Penetratin-1 because it translocates across biological membranes and allows the internalization of higher molecular weight proteins, is shown together with a non-translocating variant in which a tryptophan residue present in caveolin and all homeodomains has been replaced by a phenylalanine.

temperature, and charged phospholipids, because of the high isoelectric point of the peptides. Another important requirement for translocation is the presence of tryptophan residues, particularly at position 48 (Derossi et al., 1994). These residues are necessary to induce the formation of inverted micelles or Hexagonal phases II (J.-P. Berlose, O. Convert, D. Derossi, A. Prochiantz and G. Chassaing, unpublished results), which have been proposed to transport the peptide across the plasma membrane during its internalization (Berlose et al., 1996; Derossi et al., 1996; Prochiantz, 1996). Although the importance of the third helix was only demonstrated for Antp-HD, the structural conservation of this helix makes it likely that the mechanism demonstrated for Antp-HD applies to other homeodomain-containing polypeptides and full-length homeoproteins.

In the light of the latter findings, because caveolae are enriched in negatively charged lipids (GM1) and in cholesterol, two characteristics at the basis of homeodomain translocation, we can suppose that Engrailed bound to the microdomains translocates across the caveolar membranes, or shuttles between the interior and the exterior of the vesicles, a possibility sustained by the small but significant amount of Engrailed protected against proteinase K or trypsin (Fig. 9). Translocations across the membrane would reconcile the results of Fig. 9 (protection against proteolysis) with those showing that NaCl detaches Engrailed from the membranes (Fig. 5). Indeed, if the electrostatic interactions between Engrailed and the phospholipids facing the cytoplasmic side are disrupted in high salt, the equilibrium would be displaced in favor of the translocation of Engrailed toward the cytoplasm.

The possibility that Engrailed could translocate into the microdomains can be considered in the context of recent reports by Anderson and colleagues (Conrad et al., 1995; Smart et al., 1996). These authors have shown that interfering with cholesterol, for example by using cholesterol oxidase, provokes a transport of caveolin into the intracisternal compartment of the endoplasmic reticulum (ER). The intracisternal localization of caveolin was demonstrated both at the electron microscopic level and by experiments demonstrating a detergent-sensitive protection against proteolysis. The mechanism of the latter translocation of caveolin across the ER membranes has not been studied. One possibility is that caveolin charged with cholesterol detaches from the caveolae, reaches the ER and then translocates. Another scenario is that caveolae fuse with the ER and that caveolin translocates after this fusion.

Regardless of the mechanism involved, it is noteworthy that caveolin encompasses a sequence upstream of its membrane-inserted domain, which shows a strong similarity to the third helix of homeodomains (Fig. 10). This similarity includes a tryptophan residue necessary for the translocation of the third helix (Derossi et al., 1994, 1996). It is thus possible that the caveolin and homeoprotein translocation across membranes demonstrated by Anderson and colleagues (in the case of caveolin) and by our group (in the case of homeoproteins) occur by similar mechanisms.

In the context of the hypothesis that homeoproteins may have a paracrine activity, we thus propose that the association with microdomains could be an important step in their gaining access to a compartment that is compatible with secretion.

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REFERENCES

Akam, M. (1989). *Hox* and *HOM*: homologous gene clusters in insects and vertebrates. *Cell* **57**, 347-349.

Bally-Cuif, L. and Wassef, M. (1995). Determination events in the nervous system of the vertebrate embryo. *Curr. Opin. Genet. Dev.* **5**, 450-458.

Bedge, E. E., Hammang, J. P., Gribkoff, V. K. and Meiri, K. F. (1992). The role of GAP-43 in the molecular regulation of axon outgrowth and electrical excitability. *Persp. Dev. Neurobiol.* **1**, 21-28.

Benowitz, L. and Routtenberg, A. (1987). A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism, and synaptic plasticity. *Trends Neurosci.* **10**, 527-531.

Berlose, J., Convert, O., Derossi, D., Brunissen, A. and Chassaing, G. (1996). Conformational and associative behaviours of the third helix of antennapedia homeodomain in membrane-mimetic environments. *Eur. J. Biochem.* **242**, 372-386.

Bloch-Gallego, E., Le Roux, I., Joliot, A. H., Volovitch, M., Henderson, C. E. and Prochiantz, A. (1993). Antennapedia homeobox peptide enhances

growth and branching of embryonic chicken motoneurons in vitro. *J. Cell Biol.* **120**, 485-492.

Bouillot, C., Prochiantz, A., Rougon, G. and Allinquant, B. (1996). Axonal amyloid precursor protein expressed by neurons in vitro is present in a membrane fraction with caveolae-like properties. *J. Biol. Chem.* **271**, 7640-7644.

Briata, P., Di Blas, E., Gulisano, M., Mallamaci, A., Iannone, R., Boncinelli, E. and Corte, G. (1996). EMX1 homeoprotein is expressed in cell nuclei of the developing cerebal cortex and in the axons of the olfactory sensory neurons. *Mech. Dev*. **57**, 169-180.

Chatelin, L., Volovitch, M., Joliot, A. H., Perez, F. and Prochiantz, A. (1996). Transcription factor Hoxa-5 is taken up by cells in culture and conveyed to their nuclei. *Mech. Dev*. **55**, 111-117.

Conrad, P. A., Smart, E. J., Ying, Y.-S., Anderson, R. G. W. and Bloom, G. S. (1995). Caveolin cycles between plasma membrane caveolae and the Golgi complex by microtubule-dependent and microtubule-independent steps. *J. Cell Biol*. **131**, 1421-1433.

Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G. and Prochiantz, A. (1996). Cell internalization of the third helix of the antennapedia homeodomain is receptor-independent. *J. Biol. Chem.* **271**, 18188-18193.

Derossi, D., Joliot, A. H., Chassaing, G. and Prochiantz, A. (1994). The third helix of Antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem*. **269**, 10444-10450.

Doe, C. Q., Smouse, D. and Goodman, C. S. (1988). Control of neuronal fate by the *Drosophila* segmentation gene *even-skipped*. *Nature* **333**, 376-378.

Dotti, C. G., Parton, R. G. and Simons, K. (1991). Polarized sorting of glypiated proteins in hippocampal neurons. *Nature* **349**, 158-161.

Dotti, C. G. and Simons, K. (1990). Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell* **62**, 63-72.

Dubnau, J. and Struhl, G. (1996). RNA recognition and translational regulation by a homeodomain protein. Nature. **379**, 694-699.

Evan, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol*. **5**, 3610-3616.

Faivre-Sarrailh, C., Gennarini, G., Goridis, C. and Rougon, G. (1992). F3/F11 cell surface molecule expression in the developing mouse cerebellum at synaptic sites and within granule cells. *J. Neurosci*. **12**, 257-267.

Fra, A. M., Williamson, E., Simons, K. and Parton, R. G. (1995). *De novo* formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc. Nat. Acad. Sci. USA* **92**, 8655-8659.

Gaunt, S. J., Sharpe, P. T. and Duboule, D. (1988). Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. *Development* **104**, 169-179.

Gehring, W. J., Qian, Y. Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A. F., Resendez-Perez, D., Affolter, M., Otting, G. and Wüthrich, K. (1994). Homeodomain-DNA Recognition. *Cell* **78**, 211-223.

Gennarini, G., Cibelli, C., Rougon, G., Mattei, M.-G. and Goridis, C. (1989). The mouse cell surface protein F3: a phosphatidylinositol-anchored member of the immunoglobulin superfamily related to chicken contactin. *J. Cell Biol*. **109**, 775-788.

Gorodinsky, A. and Harris, D. A. (1995). Glycolipid-anchored proteins in neuroblastoma cells form detergent-resistant complexes without caveolin. *J. Cell Biol*. **129**, 619-627.

Green, S., Issemann, I. and Sheer, E. (1988). A versatile in vivo and in vitro eukaryotic expression vector for protein engineering. *Nucleic Acids Res.* **16**, 369.

Joliot, A., Pernelle, C., Deagostini-Bazin, H. and Prochiantz, A. (1991a). Antennapedia homeobox peptide regulates neural morphogenesis. *Proc. Nat. Acad. Sci. USA* **88**, 1864-1868.

Joliot, A. H., Triller, A., Volovitch, M., Pernelle, C. and Prochiantz, A. (1991b). α2,8-Polysialic acid is the neuronal surface receptor of Antennapedia homeobox peptide. *The New Biologist* **3**, 1121-1134.

Joyner, A. L. (1996). *Engrailed, Wnt* and *Pax* genes regulate midbrain-hindbrain development. Trends Genet. **12**, 15-20.

Keynes, R. and Krumlauf, R. (1994). Hox genes and regionalization of the nervous system. *Annu. Rev. Neurosci*. **17**, 109-132.

Kleijmeer, M. J., Raposo, G. and Geuze, H. J. (1996). Characterization of MHC class II compartments by immunoelectron microscopy. *METHODS: A Companion to Methods in Enzymology* **10**, 191-207.

Krämer, A., Haars, R., Kabisch, R., Will, H., Bautz, F.A., Bautz, E.K.F. (1980). Monoclonal antibody directed against RNA polymerase II of DM. *Mol. Gen. Genet.* **180**, 193-199.

Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.

Kurzchalia, T. V., Dupree, P., Parton, R. G., Kellner, R., Virta, H., Lehnert, M. and Simons, K. (1992). VIP21, a 21-kD membrane protein is an integral component of *trans*-Golgi-network-derived transport vesicles. *J. Cell Biol.* **118**, 1003-1014.

Le Roux, I., Duharcourt, S., Volovitch, M., Prochiantz, A. and Ronchi, E. (1995). Promoter-specific regulation of genes expression by an exogenously added homeodomain that promotes neurite growth. *FEBS Lett.* **368**, 311-314.

Le Roux, I., Joliot, A. H., Bloch-Gallego, E., Prochiantz, A. and Volovitch, M. (1993). Neurotrophic activity of the Antennapedia homeodomain depends on its specific DNA-binding properties. *Proc. Nat. Acad. Sci. USA* **90**, 9120-9124.

Lucas, W. J., Bouché-Pillon, S., Jackson, D. P., Nguyen, L., Baker, L., Ding, B. and Hake, S. (1995). Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. *Science* **270**, 1980-1983.

Miller, D. M., Shen, M. M., Shamu, C. E., Bürglin, T. R., Ruvkin, G., Dubois, M. L., Ghee, M. and Wilson, L. (1992). *C. elegans unc-4* gene encodes a homeoprotein that determines the pattern of synaptic input to specific motor neurons. *Nature* **355**, 841-845.

Mohr, E. and Richter, D. (1992). Diversity of mRNAs in the axonal compartment of peptidergic neuron in the rat. *Eur. J. Neurosci*. **4**, 870-876.

Parton, R. G. (1996). Caveolae and caveolins. *Curr. Opin. Cell. Biol.* **8**, 542-548.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.

Prochiantz, A. (1996). Getting hydrophilic compounds into cells: lessons from homeopeptides. *Curr. Opin. Neurobiol*. **6**, 629-634.

Prochiantz, A. and Théodore, L. (1995). Nuclear/growth factors. *BioEssays* **17**, 39-45.

Rijnboutt, S., Jansen, G., Posthuma, G., Hynes, J. B., Schornagel, J. H. and Strous, G. J. (1996). Endocytosis of GPI-linked membrane folate receptor-alpha. *J. Cell Biol*. **132**, 35-47.

Rivera Pomar, R., Niessing, D., Schmidt, O. U., Gehring, W. J. and Jackle, H. (1996). RNA binding and translational suppression by bicoid. *Nature* **379**, 746-749.

Rousselet, A., Autillo-Touati, A., Araud, D. and Prochiantz, A. (1990). In vitro regulation of neuronal morphogenesis and polarity by astrocyte-derived factors. *Dev. Biol*. **137**, 33-45.

Rousselet, A., Fetler, L., Chamak, B. and Prochiantz, A. (1988). Rat mesencephalic neurons in culture exhibit different morphological traits in the presence of media conditioned on mesencephalic or striatal astroglia. *Dev. Biol.* **129**, 495-504.

Rubenstein, J. R. and Puelles, L. (1994). Homeobox gene expression during development of the vertebrate brain. *Curr. Top. Dev. Biol*. **29**, 1-65.

Scherer, P. E., Okamoto, T., Chun, M., Nishimoto, I., Lodish, H. F. and Lisanti, M. P. (1996). Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. P*roc. Nat. Acad. Sci. USA* **93**, 131-135.

Skene, J. H. P. and Virag, I. (1989). Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. *J. Cell Biol.* **108**, 613-624.

Smart, E., Ying, Y., Donzell, W. and Anderson, R. (1996). A Role for Caveolin in the Transport of Cholesterol from Endoplasmic Reticulum to Plama Membrane. *J. Biol. Chem*. **271**, 29427-29435.

Song, K. S., Scherer, P. E., Tang, Z., Okamoto, T., Li, S., Chafel, M., Chu, C., Kohtz, D. S. and Lisanti, M. P. (1996). Expression of caveolin-3 in skeletal, cardiac and smooth muscle cells. *J. Biol. Chem*. **271**, 15160-15165.

Tiedge, H., Zhou, A. and Brosius, N. A. T. (1993). Transport of BC1 RNA in hypothalamo-neurohypophyseal axons. *J. Neurosci.* **13**, 4214-4219.

Trembleau, A., Morales, M. and Bloom, F. E. (1994). Aggregation of vasopressin mRNA in a subset of axonal swellings of the median eminence and posterior pituitary: light and electron microscopic evidence. *J. Neurosci.* **14**, 39-53.

Vassar, R., Chao, S. K., Sitcheran, R., Nunez, J. M., Vosshall, L. B. and Axel, R. (1994). Topographic organization of sensory projections to the olfactory bulb. *Cell* **79**, 981-991.

White, J. G., Southgate, E. and Thomson, J. N. (1992). Mutations in *Caenorhabditis elegans unc-4* gene alter the synaptic input to ventral cord motor neurons. *Nature* **355**, 838-841.

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