Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells

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Accepted 14 April 2005
Journal of Cell Science 118, 2849-2858 Published by The Company of Biologists 2005
doi:10.1242/jcs.02439

Summary

Apical plasma membrane constituents of mammalian neural stem/progenitor cells have recently been implicated in maintaining their stem/progenitor cell state. Here, we report that in the developing embryonic mouse brain, the fluid in the lumen of the neural tube contains membrane particles carrying the stem cell marker prominin-1 (CD133), a pentaspan membrane protein found on membrane protrusions of the apical surface of neuroepithelial cells. Two size classes of prominin-1-containing membrane particles were observed in the ventricular fluid: ≈600-nm particles, referred to as P2 particles, and 50-80-nm vesicles, referred to as P4 particles. The P2 and P4 particles appeared in the ventricular fluid at the very onset and during the early phase of neurogenesis, respectively. Concomitant with their appearance, the nature of the prominin-1-containing apical plasma membrane protrusions of neuroepithelial cells changed, in that microvilli were lost and large pleiomorphic protuberances appeared. P4 particles were found in various body fluids of adult humans, including saliva, seminal fluid and urine, and were released by the epithelial model cell line Caco-2 upon differentiation. Importantly, P4 particles were distinct from exosomes. Our results demonstrate the widespread occurrence of a novel class of extracellular membrane particles containing proteins characteristic of stem cells, and raise the possibility that the release of the corresponding membrane subdomains from the apical surface of neural progenitors and other epithelial cells may have a role in tissue development and maintenance. Moreover, the presence of prominin-1-containing membrane particles in human body fluids may provide the basis for a protein-based diagnosis of certain diseases.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/13/2849/DC1

Key words: extracellular membranes particles, neuroepithelium, stem cells, epithelia, prominin-1, CD133

Introduction

The proliferation versus differentiation of stem/progenitor cells is a fundamental issue of biology and medicine. Yet, the underlying cell biological mechanisms are poorly understood. A key aspect in this context is cell polarity, which has moved into the focus especially in the case of mammalian neuroepithelial cells (Chenn et al., 1998; Huttner and Brand, 1997; Wodarz and Huttner, 2003), the somatic stem/progenitor cells from which – directly or indirectly – all neurons of the central nervous system derive (Alvarez-Buylla et al., 2001; Fishell and Kriegstein, 2003; Kriegstein and Götz, 2003).

As is the case for other polarized epithelial cells, a characteristic feature of neuroepithelial cells are junctional complexes at the apical-most end of the lateral plasma membrane (Aaku-Saraste et al., 1996; Chenn et al., 1998; Ho et al., 2000; Manabe et al., 2002) and an apical plasma membrane facing a lumen (Aaku-Saraste et al., 1997; Huttner and Brand, 1997). Focusing on the latter membrane, our group recently reported that the switch of mouse neuroepithelial cells from symmetric, proliferative to asymmetric, neuron-generating cell divisions is associated with a halving of the size of the apical plasma membrane (Kosodo et al., 2004). Moreover, as hypothesized previously (Huttner and Brand, 1997), apical plasma membrane is inherited by both daughter cells upon symmetric, proliferative division of neuroepithelial cells but by only one daughter cell upon asymmetric, neuron-generating division (Kosodo et al., 2004). These studies suggest a critical role of apical plasma membrane constituents in maintaining neuroepithelial cells in a stem/progenitor cell state (Huttner and Brand, 1997; Kosodo et al., 2004; Wodarz and Huttner, 2003).

A notable constituent of the apical plasma membrane of neuroepithelial cells is prominin-1 (CD133), a pentaspan membrane glycoprotein expressed on the surface of many somatic stem cells (Alessandri et al., 2004; Corbeil et al., 2001b; Richardson et al., 2004; Weigmann et al., 1997; Yin et al., 1997). Remarkably, prominin-1 is specifically associated with plasma membrane protrusions, irrespective of the cell type

(Corbeil et al., 2001b; Corbeil et al., 2000; Giebel et al., 2004; Maw et al., 2000; Röper et al., 2000; Weigmann et al., 1997). Here, we report that, during the early stages of neurogenesis, neuroepithelial cells reduce the extent, and reorganize the nature, of their apical plasma membrane protrusions, and prominin-1-containing membrane particles appear in the lumen of the neural tube. Furthermore, the release of prominin-1-containing membrane particles by epithelial cells appears to be a widespread phenomenon.

Materials and Methods

Immunofluorescence microscopy

E7-E12.5 NMRI mouse embryos were fixed by immersion for 24 hours at 4°C in 4% paraformaldehyde, 150 mM sodium phosphate buffer, pH 7.4. (In some experiments, 1% rather than 4% paraformaldehyde was used, without any obvious difference in the results obtained.) The fixed embryos were infiltrated overnight at 4°C with 30% sucrose in phosphate buffer, embedded in TissueTek and frozen on dry-ice. Cryosections (20 μm) were collected onto HistoBond microscope slides (Paul Marienfeld GmbH, Lauda-Königshofen, Germany). The sections, dried overnight at 4°C, were hydrated with PBS and permeabilised for 15 minutes with 0.3% Triton X-100 in PBS. Residual aldehyde was quenched for 15 minutes with 50 mM NH_4Cl in PBS. Sections were blocked for 1 hour with buffer A (1% BSA, 5% fetal calf serum in PBS) and incubated overnight at 4°C in buffer A containing primary antibody against mouse prominin-1 [rat mAb 13A4 (Weigmann et al., 1997) at 8 μg/ml and rabbit antiserum αE3 (Maw et al., 2000) at 1:300 dilution]. Sections were extensively rinsed in buffer A, incubated in buffer A containing secondary antibody (affinity-purified goat anti-rat or anti-rabbit IgG conjugated either to Cy2 or Cy3), rinsed several times with buffer A, with PBS and once with distilled water, and mounted in Moviol 4.88.

Stained sections were observed using either an Olympus epifluorescence microscope with a 100× oil immersion objective connected to a CCD camera, or a Zeiss or Leica confocal laser scanning microscope. The confocal microscope settings were such that 5-μm-thick optical sections were obtained in the middle of the cryosection or at the level of the glass slide, as shown schematically in Fig. 1e. The images shown were prepared from the digital data files using Adobe Photoshop and Illustrator. For the quantification of the ring-like particles in the neural tube lumen, the area analysed in the optical sections (4-14 sections per developmental stage and brain region) was estimated using the Leica confocal software.

Isolation of prominin-1-containing membrane particles

Ventricular fluid

For each preparation, 10-30 E10.5-E13.5 NMRI mouse embryos were dissected free of embryonic membranes in ice-cold PBS. After removal of the ectodermal layer at the level of the hindbrain (E10.5-12.5) or midbrain (E13.5), the ventricular fluid (1-3 μl per embryo, depending on the age) was collected using a glass capillary connected to a micromanipulator (see Movie 1 in supplementary material). The pooled ventricular fluid (20-50 μl) was diluted with 0.5-1 ml ice-cold PBS containing protease inhibitors (Sigma, P8340; diluted 1:500). The diluted ventricular fluid was subjected to differential centrifugation as follows (all steps at 4°C): 5 minutes at 300 ***g***; supernatant, 20 minutes at 1,200 ***g***; supernatant, 30 minutes at 10,000 ***g***; supernatant, 1 hour at 110,000 ***g***. The resulting pellets (P1-P4, respectively) were resuspended in SDS sample buffer and analysed by immunoblotting for mouse prominin-1 (Corbeil et al., 2001a) using mAb 13A4 at 0.8 μg/ml.

For immunofluorescence analysis of prominin-1-containing particles in the ventricular fluid, diluted ventricular fluid from 20 E13 NMRI mouse embryos was centrifuged for 15 minutes at 10,000 ***g*** to obtain a pellet enriched in P2 particles, and the resulting supernatant for 1 hour at 200,000 ***g*** to obtain a pellet enriched in P4 particles. Pellets were fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffer, resuspended in fixative, spotted onto polylysine-coated glass slides, allowed to dry, and subjected to immunofluorescence analysis as described above for cryosections.

For immunogold labelling and negative staining electron microscopy of prominin-1-containing particles in the ventricular fluid, diluted ventricular fluid from 10-20 E10.5-11.5 NMRI mouse embryos was subjected to differential centrifugation to obtain P1-P4 pellets. The P2 and P4 pellets were fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffer and processed for electron microscopy as described below.

Body fluids

Human seminal fluid, saliva, urine and lacrimal fluid were obtained from healthy volunteers with informed consent. Protease inhibitors were added to each sample. Seminal fluid (2-3 ml) was kept for 30 minutes at room temperature to allow liquefaction. Saliva (2 ml) was mixed with an equal volume of ice-cold PBS. Both seminal fluid and saliva were then filtered through gauze. Urine (4 ml) was used directly. Lacrimal fluid (50 μl) was collected with a Pasteur pipette after stimulation with onion juice. Samples were subjected to differential centrifugation as follows: 5 minutes at 300 ***g***; supernatant, 30 minutes at 10,000 ***g***; supernatant, 1 hour at 200,000 ***g***; supernatant, 1 hour at 400,000 ***g***. The resulting pellets were resuspended in SDS sample buffer, and aliquots corresponding to 25-400 μl original fluid were analysed by immunoblotting for human prominin-1 using rabbit antiserum hE2 (Florek et al., 2005) (1:3000-1:5000).

Caco-2 cells

Caco-2 cells were grown at 37°C in a 5% CO_2 atmosphere in modified Eagle's medium (MEM) supplemented with 20% fetal calf serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The medium was changed every 2-3 days.

For immunoblot analysis of prominin-1-containing particles in the medium, Caco-2 cells grown for 9 days post-confluency received fresh medium, which was collected after 24 hours (24 h-conditioned medium). The conditioned medium was subjected to differential centrifugation as described above for ventricular fluid, followed by immunoblotting of the resulting P1-P4 pellets for human prominin-1 using rabbit antiserum hE2.

For the isolation of P4 particles from Caco-2-cell-conditioned medium, cells grown for 14 days post-confluency received fresh, serum-free medium (OptiMEM, Gibco-BRL) otherwise supplemented as above. After 24 hours, the conditioned medium was centrifuged for 30 minutes at 10,000 ***g*** and the resulting supernatant (30 ml) concentrated to 0.5 ml using Centricon plus-20 (Millipore). The sample was subjected to equilibrium sucrose gradient (50-800 mM) centrifugation as described previously (Huttner et al., 1983). Fraction 7, containing part of the peak of prominin-1 as revealed by immunoblotting (see Fig. 6b below), was diluted 1:2 with PBS and centrifuged for 30 minutes at 110,000 ***g*** onto a 20-μl 800 mM sucrose cushion, and the bottom 30 μl were analysed by electron microscopy.

For the differentiation experiments, 600,000 cells/cm^2 were plated onto 5-cm dishes. Cells received fresh medium 16 hours after plating, when they typically had reached confluency (t=0 post-confluency). Cells were grown for up to 21 days post-confluency and received fresh medium 24 hours before analysis. The 24-hour-conditioned medium was centrifuged for 30 minutes at 10,000 ***g*** and the resulting supernatant for 1 hour at 110,000 ***g*** to obtain the P4 pellet. In parallel,

the cell monolayer was washed with ice-cold PBS and the cells were lysed for 30 minutes at 4°C in 200 µl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0) containing protease inhibitors. The cell lysate was centrifuged for 15 minutes at 10,000 ***g*** and the resulting supernatant analysed. In some experiments, cells were directly dissolved into SDS sample buffer. Cell lysates and P4 pellets were analysed by immunoblotting for human prominin-1 using rabbit antiserum hE2 and for α-tubulin using a mouse mAb (Sigma, T-9026; 1:5000). Chemiluminescence was detected using Hyperfilm (Amersham) and quantified after scanning using MacBAS software.

Electron microscopy

Neuroepithelium

E8.5-11.5 NMRI mouse embryos were fixed overnight at 4°C in 4% paraformaldehyde in phosphate buffer and, in some experiments, additionally in 4% paraformaldehyde, 0.05% glutaraldehyde in 100 mM sodium phosphate buffer pH 7.4. Fixed embryos were cut into pieces containing defined parts of the central nervous system, which were infiltrated with gelatine at 37°C, embedded in gelatine and infiltrated with sucrose at 4°C, then cryosectioned as described previously (Wucherpfennig et al., 2003). After removal of the gelatine from the cryosections by incubation on a drop of PBS at 37°C, they were subjected to immunolabelling (Wucherpfennig et al., 2003), using mAb 13A4 (25-50 µg/ml) followed by 12-nm gold-coupled secondary antibody (Dianova, Hamburg, Germany) or, in some experiments, rabbit antiserum to rat IgG (Cappel #55704) followed by protein A/10-nm gold. Cryosections were contrasted for 10 minutes on ice with a mixture of 1.9% methyl cellulose/0.3% uranyl acetate. Samples were viewed in a Morgagni electron microscope (FEI, Eindhoven, The Netherlands).

P2 and P4 particles from ventricular fluid

Fixed P2 and P4 pellets were resuspended in fixative, adsorbed to 400-mesh formvar/carbon-coated grids, processed through immunolabelling for prominin-1 as described above, and subjected to negative contrasting using a mixture of 1.9% methyl cellulose plus either 0.1% uranyl acetate (P2) or 0.3% uranyl acetate (P4).

P4 particles from Caco-2 cells

The particles in suspension were adsorbed to 400-mesh formvar/carbon-coated grids, fixed with 4% paraformaldehyde in phosphate buffer, subjected to either single-immunolabelling using the mouse mAb AC133 against human prominin-1 (10 µg/ml; Miltenyi, Biotec, Bergisch Gladbach, Germany) or double-immunolabelling using rabbit antiserum hE2 against human prominin-1 (Florek et al., 2005) (1:150) and mouse mAb CLB-gran/12, 425 against CD63 (10 µg/ml; Sanquin, Amsterdam, The Netherlands), followed by 6-nm gold-coupled anti-rabbit and 12-nm gold-coupled anti-mouse secondary antibodies (Dianova), and negatively contrasted using a mixture of 1.9% methyl cellulose and 0.3% uranyl acetate.

Scanning electron microscopy

E11.5 NMRI mouse embryos were fixed overnight at 4°C in 4% paraformaldehyde, 2% glutaraldehyde in 150 mM sodium phosphate buffer pH 7.4. Fixed embryos were dehydrated in ascending concentrations of acetone (30%, 50%, 70%, 90% and 100%) and subjected to critical-point drying using liquid carbon dioxide (CPD 030; BAL-TEC, Witten, Germany). The samples were mounted on aluminium stubs, coated with gold in a sputter coater (SCD 050, BAL-TEC) and viewed in a scanning electron microscope (XL 30 ESEM FEG, FEI).

Results

Occurrence of membrane particles carrying the stem cell marker prominin-1 in the ventricular fluid of the embryonic mouse brain

Immunofluorescence analysis of the developing mouse brain revealed that prominin-1 immunoreactivity was not only concentrated at the apical surface of the neuroepithelium, as observed previously (Kosodo et al., 2004; Weigmann et al., 1997), but was also associated with particles present in the ventricular fluid (Fig. 1). These prominin-1-containing particles escaped detection when optical sections were taken by confocal microscopy in the middle of the cryosection (Fig. 1a,b,e), but were readily observed in optical sections taken at the bottom of the cryosection (Fig. 1c-e). Presumably, only those membrane particles present in the lumen of the neural tube at the time of fixation were recovered with the cryosection at the end of the immunofluorescence procedure that sedimented onto, and adsorbed to, the glass slide. The prominin-1-containing particles in the ventricular fluid were observed with two different antibodies against distinct

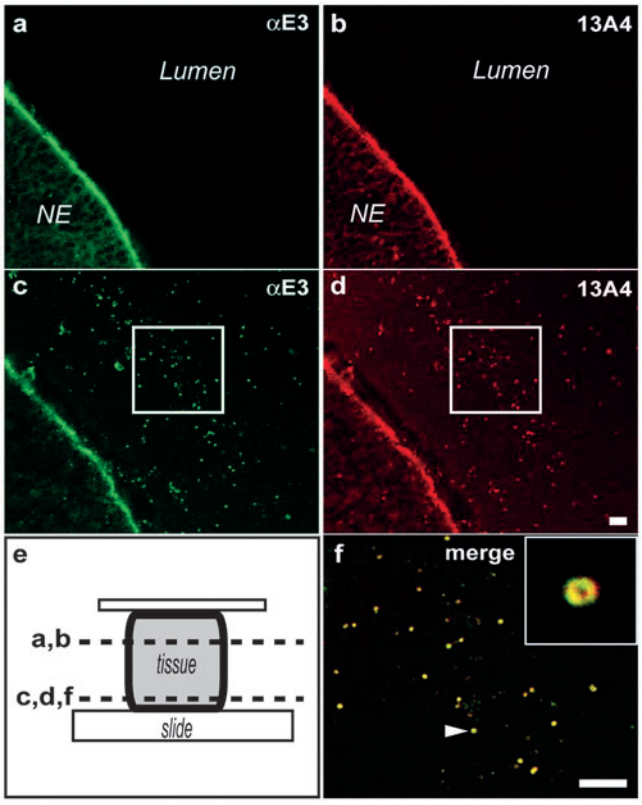

Fig. 1. Occurrence of prominin-1-containing particles in the ventricular fluid of the embryonic mouse brain. Double immunofluorescence of a cryosection (20 µm) of an E11.5 mouse embryo using mAb 13A4 (b,d,f, red) and antiserum αE3 (a,c,f, green) directed against distinct extracellular epitopes of prominin-1. Single 5-µm optical sections of the midbrain were obtained by confocal microscopy in the middle of the cryosection (a,b) and at the level of the glass slide (c,d,f), as outlined in e. (f) Merged images of the area indicated by the white squares in c and d; note the co-localisation of the 13A4 and αE3 immunoreactivities. Inset in f is a higher magnification of the particle indicated by the arrowhead in f. Bars, 10 µm.

extracellular epitopes of prominin-1 (Fig. 1c,d,f) and often had a ring-like appearance (Fig. 1f inset). They lacked immunoreactivity for cadherin, a protein on the lateral plasma membrane of neuroepithelial cells (Aaku-Saraste et al., 1996; Kosodo et al., 2004; Nose and Takeichi, 1986), and only few of them were weakly stained for actin (Fig. S1 in supplementary material).

The physiological occurrence of prominin-1-containing particles in the lumen of the neural tube was corroborated by biochemistry. Ventricular fluid was collected from E10.5-11.0 mouse embryos by introducing, after removal of the ectodermal layer, a glass capillary into the neural tube lumen at the level of the hindbrain, followed by gentle aspiration of the liquid (see Movie1 in supplementary material). Differential centrifugation of the ventricular fluid followed by immunoblotting for prominin-1 revealed the existence of two distinct populations of prominin-1-containing particles, which were largely recovered in the P2 pellet after centrifugation for 20 minutes at 1,200 ***g*** and in the P4 pellet after centrifugation for 1 hour at 110,000 ***g***, respectively (Fig. 2a).

The prominin-1-containing particles found in the P2 and P4 pellets were isolated from E13 ventricular fluid (centrifugation for 15 minutes at 10,000 ***g*** and 1 hour at 200,000 ***g***, respectively, to increase the yield) and analysed by immunofluorescence for prominin-1. This showed that the particles with a ring-like appearance observed in the neural tube lumen (Fig. 1) were recovered in the P2 fraction (Fig. 2b) and were substantially larger than the particles recovered in the P4 fraction (Fig. 2c), consistent with the ***g*** force required for their sedimentation (Fig. 2a). Both populations of prominin-1-containing particles, from now on referred to in short as P2 and P4 particles, appeared to be relatively homogeneous.

Upon immunogold electron microscopy (EM) for prominin-1, the P2 particles isolated from E11.5 ventricular fluid appeared as electron-dense membrane structures with an average diameter of 600±100 nm, which showed abundant prominin-1 immunoreactivity on their surface (Fig. 2e,f). By contrast, the P4 particles appeared as ≈50-80-nm membrane vesicles with prominin-1 surface labelling (Fig. 2d).

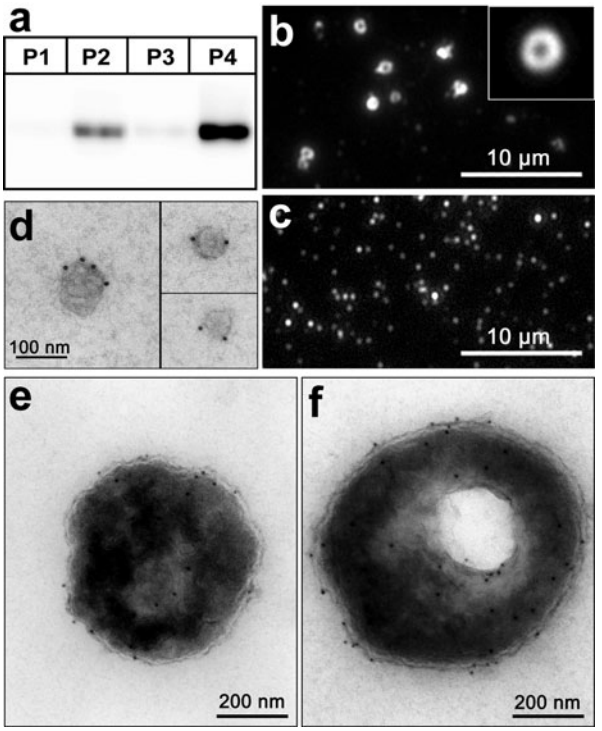

Fig. 2. Isolation of large and small prominin-1-containing membrane particles from the embryonic ventricular fluid. (a) Ventricular fluid of E10.5-E11 mouse embryos was subjected to differential centrifugation for 5 minutes at 300 ***g*** (P1), 20 minutes at 1,200 ***g*** (P2), 30 minutes at 10,000 ***g*** (P3) and 1 hour at 110,000 ***g*** (P4). The entire P1-P4 pellets were analysed by immunoblotting for prominin-1. (b,c) Immunofluorescence of prominin-1 of P2 and P4 particles obtained by differential centrifugation of E13 ventricular fluid for 15 minutes at 10,000 ***g*** (b) and 1 hour at 200,000 ***g*** (c), respectively. The inset in b shows a P2 particle at higher magnification, which is very similar to the one shown in the inset of Fig. 1f. (d-f) Negative staining EM of prominin-1 immunogold labelled P4 (d) and P2 (e,f) particles obtained by differential centrifugation of E11.5 ventricular fluid as in a.

Differential appearance of prominin-1-containing P2 and P4 particles in the ventricular fluid during the development of the embryonic mouse brain

Immunoblotting for prominin-1 of ventricular fluid collected at various embryonic stages (E10.5-E13.5) showed a rise in total prominin-1-containing particles at early stages (E10.5-E12.5) followed by a decline thereafter (E12.5-E13.5) (Fig. 3a,

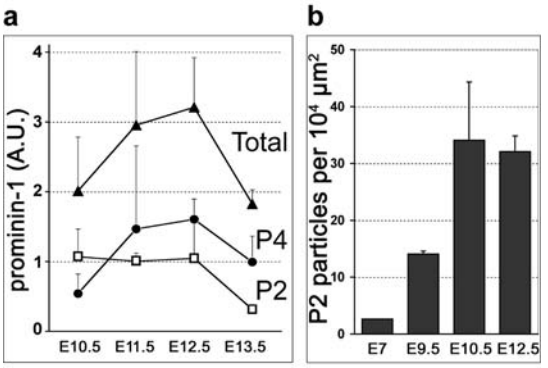

Fig. 3. Time course of appearance of P2 and P4 prominin-1-containing membrane particles in the ventricular fluid during embryonic mouse brain development. (a) Ventricular fluid of E10.5-E13.5 mouse embryos was subjected to differential centrifugation as in Fig. 2a followed by immunoblotting of the pellets for prominin-1. Total prominin-1 immunoreactivity (sum of P1-P4 pellets, triangles) and prominin-1 immunoreactivity in the P2 pellet (squares) and the P4 pellet (circles) at the various developmental stages was determined. Data are the mean of three independent experiments; bars indicate s.d. For any given experiment, the same volume of ventricular fluid was analysed at the various developmental stages. (b) Cryosections of E7-E12.5 mouse embryos were subjected to immunofluorescence for prominin-1. The number of the ring-like prominin-1-containing particles (see inset in Fig. 1f) in the ventricular fluid was quantified per unit area in single optical sections at the level of the glass slide. E7, amniotic fluid near the neural plate (19×10^4 μm^2); E9.5-E12.5, mean of forebrain and hindbrain ventricular fluids, bars indicate the variation of the forebrain/hindbrain values from the mean (E9.5, 66×10^4 μm^2; E10.5, 139×10^4 μm^2; E12.5, 134×10^4 μm^2).

triangles). The rise in total prominin-1 immunoreactivity was due to an increase in the prominin-1 recovered in the P4 pellet (reflecting an increase in either the number of P4 particles or the concentration of prominin-1 on them), while the prominin-1 recovered in the P2 pellet remained largely constant. The subsequent decline of total prominin-1 immunoreactivity was

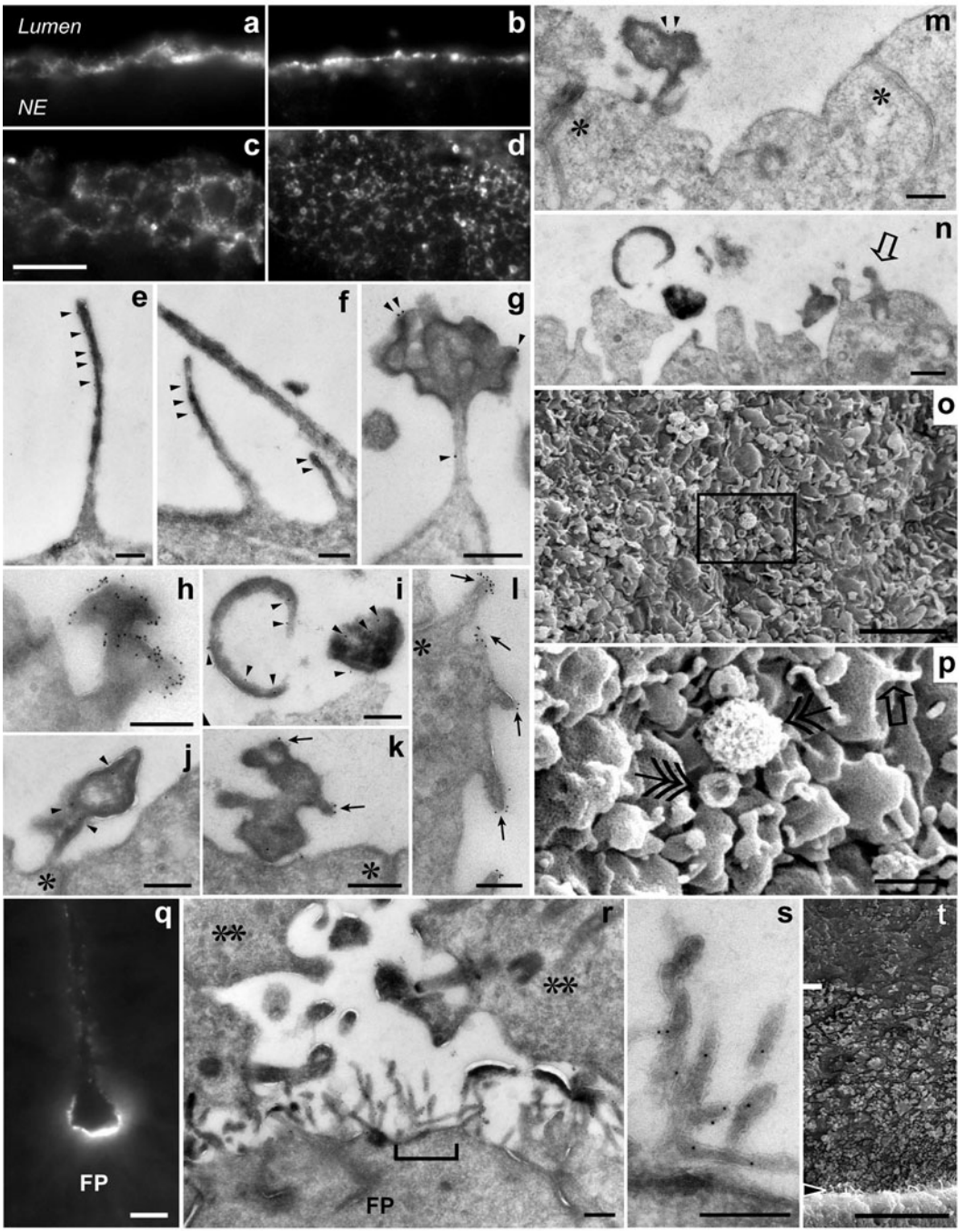

Fig. 4. Prominin-1-containing protrusions on the apical plasma membrane of neuroepithelial cells at various stages of embryonic mouse brain development. (a-d) Prominin-1 immunofluorescence at the apical surface of neuroepithelial cells at E8.5 (a,c; future hindbrain) and E11.5 (b,d; dorsal telencephalon), viewed from the lateral (a,b) and lumenal (c,d) side of the neuroepithelium. Scale bar in c: 10 µm. (e-n) Prominin-1 immunogold EM of the apical plasma membrane of neuroepithelial cells at E8.5 (e-g) and E11.5 (h-n) (h,i,k,l,n: midbrain; j,m: forebrain). Scale bars: 300 nm. Some of the gold particles (12 nm) are indicated by arrowheads and junctional complexes by asterisks. (e,f) Microvilli. (g-k) Large pleiomorphic protuberances; note the thin stalks (g,j), the vicinity of the junctional complexes (j,k), the abundant prominin-1 immunoreactivity (h), and the small prominin-1-labelled membrane buds (arrows) in (k); (i) shows the prominin-1-labelled cup-shape surface structure of (n) at higher magnification. (l) Short, thin protrusions; note the concentration of prominin-1 immunoreactivity at the tips (arrows). (m,n) Overviews of the apical plasma membrane of a single (m) and several (n) neuroepithelial cells; note the prominin-1-labelled protuberance in m and the cilium (arrow, note the basal body), but lack of microvilli, in n. (o,p) Scanning EM of the apical plasma membrane of E11.5 neuroepithelial cells in the forebrain. The boxed region in o indicates the area shown at higher magnification in p. Open arrow, cilium; double-headed arrow, cauliflower-like protuberance; triple-headed arrow, cup-shape protuberance. Scale bar: 5 µm (o); 1 µm (p). (q-t) Floorplate of the E11.5 spinal cord. (q) Prominin-1 immunofluorescence at relatively low exposure to show the more intense immunoreactivity of the floorplate cells (FP). Scale bar: 10 µm. (r) Overview of prominin-1 immunogold EM; note the microvilli of the floorplate cells (FP) and the pleiomorphic protuberances but lack of microvilli on the neuroepithelial cells adjacent to the floor plate (double-asterisks); the bracket indicates the area shown at higher magnification in s. Scale bar: 300 nm. (s) Prominin-1-labelled microvilli of the floorplate (12-nm gold). Scale bar: 300 nm. (t) Scanning EM of the apical plasma membrane. Note the bending of the lumenal surface, resulting in a lateral view of the microvilli-bearing floorplate cells and a lumenal view of the adjacent neuroepithelial cells. The black triangle and white bar at the left margin indicate the transition from floorplate to adjacent neuroepithelial cells and an apparent boundary between neuroepithelial cells with abundant vs. sparse protrusions, respectively. Bar, 10 µm.

due to a decrease of prominin-1 in both, the P2 and P4 pellet (Fig. 3a, squares and circles, respectively).

Given that at the earliest developmental stage studied by immunoblotting, E10.5, the bulk of prominin-1 was associated with P2 particles, we chose immunofluorescence for prominin-1 on cryosections (see Fig. 1) to obtain information about their appearance in the ventricular fluid at stages earlier than E10.5, when the isolation and biochemical analysis of this fluid is not feasible. This showed the progressive appearance of the P2 particles in the ventricular fluid concomitant with the transition from the neural plate (E7) to the neural tube and the onset of neurogenesis (E9.5-E10.5; Fig. 3b). Between E10.5 and E12.5, the concentration of P2 particles in the ventricular fluid as determined by prominin-1 immunofluorescence remained constant (Fig. 3b). Together with the results of immunoblotting (Fig. 3a, squares), this also indicates that there was no significant change in the concentration of prominin-1 per P2 particle between E10.5 and E12.5.

Analysis of the prominin-1-containing protrusions of the apical plasma membrane of neuroepithelial cells during early development of the embryonic mouse brain

In light of the developmental regulation of the appearance of prominin-1-containing membrane particles in the ventricular fluid (Fig. 3), we investigated the morphology of apical plasma membrane protrusions of neuroepithelial cells before (E8.5) and after (E11.5) the onset of neurogenesis. While a lateral view of the neuroepithelium did not reveal a major difference in the prominin-1 immunostaining of the apical surface at the two developmental stages (Fig. 4a,b), a view onto the apical surface from the lumen did. At E8.5, prominin-1 immunoreactivity showed a fine punctate pattern (Fig. 4c), whereas at E11.5, the pattern was dominated by larger structures that often had a ring-like appearance (Fig. 4d), reminiscent of the P2 particles found in the ventricular fluid (Fig. 1f inset, Fig. 2b).

At the EM level, the predominant prominin-1-labelled structures of the apical plasma membrane of E8.5 neuroepithelial cells were microvilli (Fig. 4e,f), consistent with earlier prominin-1 immunogold EM data (Weigmann et al., 1997) and with previous studies showing the presence of abundant microvilli in rodent neural plate neuroepithelial cells (Shepard et al., 1993). By contrast, at E11.5, the apical surface of neuroepithelial cells largely lacked microvilli (Fig. 4m,n), and prominin-1 surface immunoreactivity was mostly associated with large electron-dense pleiomorphic protuberances that emerged from the apical plasma membrane via a thin stalk (Fig. 4h-k). Similar protuberances were already detected at E8.5 (Fig. 4g), albeit at much lower abundance. In addition to these large protuberances, prominin-1 immunoreactivity at E11.5 was associated with short, thin apical plasma membrane protrusions (Fig. 4l). Scanning EM of the apical surface of E11.5 neuroepithelial cells corroborated this morphology and in addition confirmed the presence of cilia (Nonaka et al., 1998) (Fig. 4n,p).

Persistence of prominin-1-containing microvilli after the onset of neurogenesis in the floorplate

A notable exception with regard to the lack of apical microvilli after the onset of neurogenesis was the floorplate. At E11.5, consistent with the strong prominin-1 immunofluorescence of the floorplate (Fig. 4q), the corresponding cells showed abundant prominin-1-containing microvilli using immunogold EM (Fig. 4r,s). By contrast, in both transmission (Fig. 4r) and scanning (Fig. 4t) EM, the apical surface of the adjacent neuroepithelial cells was dominated by the presence of large pleiomorphic protuberances.

Occurrence of prominin-1-containing particles in various body fluids

Given the widespread occurrence of prominin-1 in various embryonic and adult tissues (Corbeil et al., 2001b; Corbeil et al., 2000; Weigmann et al., 1997), we investigated whether prominin-1-containing particles are present in body fluids other than the ventricular fluid of the developing brain. Examination of various body fluids of adult humans by differential centrifugation followed by immunoblotting revealed the occurrence of prominin-1-containing particles in seminal fluid, saliva, urine and lacrimal fluid (Fig. 5). In all of these body fluids (except perhaps lacrimal fluid), the prominin-1-containing particles were of the P4 type as they were sedimented by centrifugation for 1 hour at 200,000 ***g*** but not 30 minutes at 10,000 ***g***. (It remains to be investigated whether the sedimentation of the prominin-1-containing particles in lacrimal fluid after 30 minutes at 10,000 ***g*** reflects a larger size or some kind of membrane aggregation.)

Prominin-1-containing P4 particles lack the exosomal marker CD63

Given that prominin-1 is endogenously expressed by Caco-2 cells (Corbeil et al., 2000), a human colon carcinoma-derived cell line, we explored the use of these cells as a model to study the nature and release of prominin-1-containing particles from epithelial cells into the extracellular fluid. Indeed, analysis of 24-hour-conditioned medium of Caco-2 cells, grown for 10 days post-confluency, by differential centrifugation followed by immunoblotting showed that these cells released prominin-1-containing particles into the medium (Fig. 6a). These particles were mostly of the P4 type, being sedimented by centrifugation for 1 hour at 110,000 ***g*** but not 20 minutes at 1,200 ***g*** (Fig. 6a). Upon further fractionation by equilibrium

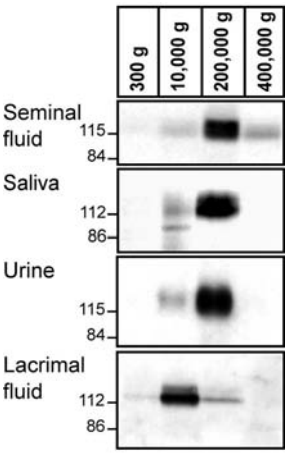

Fig. 5. Occurrence of prominin-1-containing membrane particles in various human body fluids. Various human body fluids were subjected to differential centrifugation for 5 minutes at 300 ***g*** (300 ***g***), 30 minutes at 10,000 ***g*** (10,000 ***g***), 1 hour at 200,000 ***g*** (200,000 ***g***) and 1 hour at 400,000 ***g*** (400,000 ***g***). The resulting pellets were analysed by immunoblotting for prominin-1.

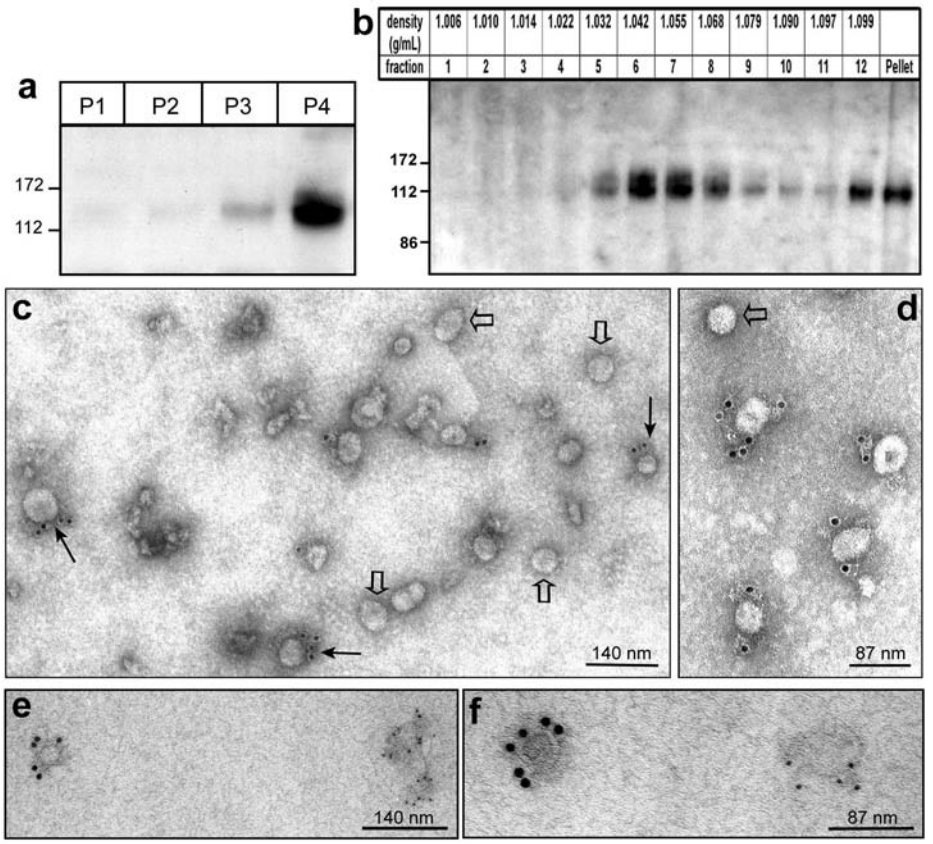

Fig. 6. The P4 prominin-1-containing membrane particles are distinct from exosomes carrying the tetraspanin CD63. (a) 24-hour-conditioned medium of Caco-2 cells grown for 10 days post-confluency was subjected to differential centrifugation as in Fig. 2a, and the resulting pellets were analysed by immunoblotting for prominin-1. (b) 24-hours-conditioned medium of Caco-2 cells grown for 14 days post-confluency was centrifuged for 30 minutes at 10,000 ***g***, and the resulting supernatant, containing the P4 particles, was concentrated and subjected to equilibrium sucrose gradient centrifugation. Aliquots of the fractions (1.5%) and the pellet (50%) were analysed by immunoblotting for prominin-1. (c,d) Negative staining and prominin-1 immunogold (12 nm) labelling EM of the P4 particles present in fraction 7 of the sucrose gradient. Note the presence of both prominin-1-positive (arrows, indicated only in c) and prominin-1-negative (open arrows) small vesicles. (e,f) Negative staining EM of prominin-1 (6-nm gold) and CD63 (12-nm gold) double-immunogold labelled particles present in fraction 7 of the sucrose gradient. Note that the small vesicles showing prominin-1 immunoreactivity are distinct from those showing CD63 immunoreactivity.

sucrose gradient centrifugation, the prominin-1-containing P4 particles (as revealed by immunoblotting) were found in a discrete peak of relatively low buoyant density (Fig. 6b), similar to that of synaptic vesicles (Huttner et al., 1983).

Interestingly, immunogold labelling followed by negative staining EM showed that only a subpopulation of the small, ≈50-80-nm membrane vesicles present in the peak fraction of the sucrose gradient contained prominin-1, while other, similarly small vesicles lacked prominin-1 immunoreactivity on their surface (Fig. 6c,d). This second, distinct subpopulation of small prominin-1-negative membrane vesicles, but not the small prominin-1-positive membrane vesicles (Fig. 6e,f, small gold particles), were immunoreactive for CD63 (Fig. 6e,f, large gold particles), a tetraspan membrane protein highly enriched in exosomes from virtually any cell type (Théry et al., 2002; van Niel et al., 2001). These data indicate that the small prominin-1-containing P4 particles are distinct from exosomes.

Release of prominin-1-containing P4 particles by Caco-2 cells upon differentiation

Caco-2 cells are known to undergo enterocytic differentiation several days after reaching confluency (Louvard et al., 1992; Pinto et al., 1983). Given that the prominin-1-containing P4 particles in the ventricular fluid increased concomitant with the progression of neurogenesis (Fig. 3a,b), i.e. the differentiation of neuroepithelial cells (Alvarez-Buylla et al., 2001; Haubensak et al., 2004; Kriegstein and Götz, 2003), it was of interest to investigate whether the release of the prominin-1-containing P4-type particles from Caco-2 cells into the medium might be related to their differentiation. We therefore analysed Caco-2 cells and the medium conditioned for 24 hours by these cells at various time points during a 21-day period after the cells had reached confluency (Fig. 7). Analysis of the P4 pellets prepared from the media by immunoblotting for prominin-1 showed that the release of P4 particles by Caco-2 cells into the medium increased steeply between 6 and 8 days post-confluency (Fig. 7a, top), consistent with a possible relationship between P4 particle release and the differentiation of Caco-2 cells. The prominin-1 released by the cells as P4 particles at day 8 post-confluency amounted to ≈10% of total (Fig. 7b). In line with previous observations (Florek et al., 2005), the level of cell-associated prominin-1 between day 1 and day 8 post-confluency remained constant (Fig. 7c) and decreased by ≈25% at day 21 post-confluency (data not shown).

Discussion

The present study reports the existence of novel extracellular membrane particles that carry a surface marker of stem cells, prominin-1 (CD133). Although prominin-1 expression in embryonic and adult tissues has previously been studied by immunohistochemistry (Corbeil et al., 2000; Kosodo et al., 2004; Weigmann et al., 1997), these particles have escaped detection, presumably because of their small size and/or their loss from the tissue section during the immunohistochemical procedure.

The prominin-1-containing membrane particles in the ventricular fluid, described here, lacked other plasma

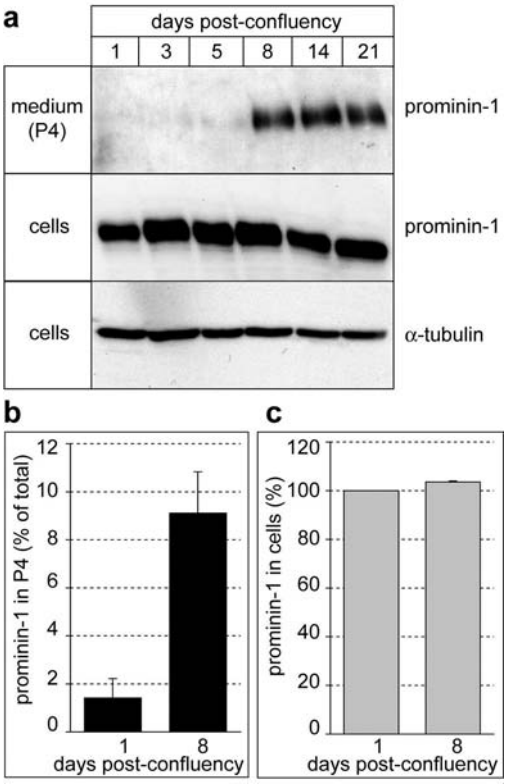

Fig. 7. Appearance of prominin-1-containing membrane particles in the culture medium upon differentiation of Caco-2 cells. Caco-2 cells were grown for up to 21 days post-confluency. Cells received fresh medium 24 hours before analysis. At the indicated time points, cells and the P4 pellet obtained from the conditioned medium were analysed by immunoblotting. (a) Immunoblots. Top, prominin-1 in the P4 pellet of the medium; middle, prominin-1 in the cells; bottom, α-tubulin in the cells. (b,c) Quantitation of immunoblots for day 1 and day 8 post-confluency. (b) Prominin-1 immunoreactivity in the P4 pellet of the medium is expressed as percentage of total (sum of cells plus P4). Data are the mean of three independent experiments; bars indicate s.d. (c) Prominin-1 immunoreactivity in the cells was normalized to that of α-tubulin. Normalized prominin-1 immunoreactivity in the cells at day 8 post-confluency is expressed relative to that at day 1, which was arbitrarily set to 100%. Data are the mean of two independent experiments; bars indicate the variation of the individual values from the mean.

membrane markers such as cadherin, and only few of them were weakly immunostained for actin. These observations suggest that the appearance of prominin-1-containing membrane particles in the extracellular medium does not simply reflect indiscriminate cell fragmentation but, rather, a specific release process. The latter conclusion is also supported by our findings (unpublished data) that prominin-1 associated with the P4 particles released by Caco-2 cells showed the same detergent solubility/insolubility and cholesterol dependence that is characteristic for prominin-1 associated with apical plasma membrane protrusions and distinguishes it from other membrane proteins that are associated with the planar regions of the apical plasma membrane (Röper et al., 2000).

Importantly, various lines of evidence indicate that neither of the two size classes of prominin-1-containing membrane particles described here are exosomes, i.e. the internal membrane vesicles of multivesicular bodies that are released into the extracellular space by exocytosis (Février and Raposo, 2004; Stoorvogel et al., 2002; Théry et al., 2002), a process proposed to occur in the floorplate neuroepithelium (Tanaka et al., 1988). The P2 particles, with an average diameter of 600 nm, are much larger than exosomes, which typically have a diameter of 50-90 nm (Février and Raposo, 2004; Stoorvogel et al., 2002; Théry et al., 2002). The prominin-1-containing P4 particles, though similar in size to exosomes, in fact represented a subpopulation of small membrane vesicles distinct from exosomes, lacking immunoreactivity for CD63, a tetraspan membrane protein highly enriched in exosomes from virtually any cell type (Théry et al., 2002). Moreover, prominin-1 has been characteristically found on protrusions of the plasma membrane (Corbeil et al., 2001b) (rather than in multivesicular bodies), which also does not support the prominin-1-containing P4 particles being multivesicular body-derived exosomes.

We observed two types of prominin-1-containing protrusions of the apical plasma membrane of neuroepithelial cells that would be consistent with them being the presumptive site of formation of P2 and P4 particles. One type are the previously described microvilli (Weigmann et al., 1997). Microvilli have been proposed to give rise to small membrane vesicles (Beaudoin and Grondin, 1991; Hobbs, 1980), and prominin-1-containing membrane buds of the size of P4 particles emerge from the microvilli of neuroepithelial cells [see Fig. 2c in Weigmann et al. (Weigmann et al., 1997)]. It is therefore possible that the prominin-1-containing 50-80-nm P4 particles in the ventricular fluid originate from microvilli, as well as other small protrusions, of the apical plasma membrane of neuroepithelial cells (including the cells of the floorplate, see below).

The other type of prominin-1-containing protrusions are large pleiomorphic protuberances that are connected to the apical plasma membrane proper via a thin stalk. Their appearance resembled that of the prominin-1-containing 600-nm P2 particles in the ventricular fluid, and hence the latter membrane particles may arise from these apical protuberances of neuroepithelial cells. Given the likely origin of the novel extracellular membrane particles described here from plasma membrane protrusions (i.e. from ‘prominent’ structures), and the apparent enrichment of prominin-1, a stem cell marker (Alessandri et al., 2004; Corbeil et al., 2001b; Richardson et al., 2004; Weigmann et al., 1997; Yin et al., 1997), in these particles, it may also be appropriate to refer to the P2 and P4 particles as large and small ‘prominosomes’, respectively.

The abundance of microvilli and large protuberances of the apical neuroepithelial cell surface underwent a striking change during embryonic development. While prominin-1-containing microvilli were abundant at an early stage, i.e. the neural plate stage, this was no longer the case at later stages, i.e. after neural tube closure and the onset of neurogenesis, with one notable exception, the floorplate (see below). Conversely, the abundance of the pleiomorphic large protuberances on the apical surface of neuroepithelial cells increased during embryonic brain development from E8.5 to E11.5. It is interesting to note that this increase coincided with the appearance of the prominin-1-containing P2 particles in the ventricular fluid, consistent with these membrane particles originating from the apical protuberances.

The developmental regulation of the microvilli and large protuberances of the apical neuroepithelial cell surface on the one hand, and of the P4- and P2-type prominin-1-containing membrane particles in the ventricular fluid on the other, is intriguing. At the onset of neurogenesis, neuroepithelial cells switch from symmetric, proliferative to asymmetric, neuron-generating divisions (Haubensak et al., 2004; Miyata et al., 2001; Noctor et al., 2001), transform into radial glial cells (Götz, 2003; Kriegstein and Götz, 2003) and give rise to neuron-generating basal progenitors (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). All these processes are accompanied by a reduction in size of (Kosodo et al., 2004), or complete loss of (Miyata et al., 2004), apical plasma membrane, which may well involve the release into the ventricular fluid of the prominin-1-containing membrane particles described in the present study.

Despite this potential relationship between the release of prominin-1-containing membrane particles and neural development, it should be emphasized that the physiological significance of this release is presently unclear and remains to be investigated. Two principal roles of these particles and their release from neuroepithelial (as well as other) cells may be considered. First, prominin-1-containing membrane particles may function in intercellular communication. In this context, it is worth noting that the small, P4-type prominin-1-containing membrane particles in the ventricular fluid increase during a period (E10.5-E12.5) when apical microvilli are most abundant in the floorplate. Given the role of the floorplate as a signalling centre (Dodd et al., 1998; Marti and Bovolenta, 2002; Marti et al., 1995), this not only raises the possibility that the P4 particles, at least at these stages of central nervous system development, originate from the floorplate, but also that they may exert a signalling role.

Second, the release of prominin-1-containing membrane particles by cells may be a means of disposal of membrane microdomains that endow these cells with certain properties. For example, given that prominin-1 is a stem cell marker (Alessandri et al., 2004; Corbeil et al., 2001b; Richardson et al., 2004; Weigmann et al., 1997; Yin et al., 1997), the release of prominin-1-containing membrane particles from cells may contribute to a down-regulation of their stem/progenitor cell properties, or to their differentiation. The developmental regulation of the prominin-1-containing membrane particles in the ventricular fluid during neurogenesis, as well as their release concomitant with the differentiation of Caco-2 cells, are consistent with (but do not prove) such a role.

Finally, the widespread occurrence of prominin-1-containing membrane particles in various human body fluids deserves comment. These body fluids constitute an easily accessible source of material for a protein-based diagnosis of human diseases involving prominin-1 (see Maw et al., 2000). As a broader perspective, quantitative and qualitative analyses of the prominin-1-containing membrane particles in such human body fluids may provide relevant medical information about the epithelial tissues concerned.

We thank Nicola Bauer for help with scanning EM, and members of the Corbeil and the Huttner groups for help and discussions. V.D. was supported by the European Molecular Biology Organisation (ALTF 258-2003). D.C. was supported by the Deutsche Forschungsgemeinschaft (SPP 1109, CO 298/2-1; SFB/TR13-04 B1) and W.B.H. by the Deutsche Forschungsgemeinschaft (SPP 1109, Hu 275/7-2; SPP 1111, Hu 275/8-2; SFB/TR13-04 B1) and the Fonds der Chemischen Industrie.

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