INTRODUCTION

The proliferating CNS precursor cells of the developing neural tube are organized as a columnar epithelium of neuroectodermal cells spanning from the inner, ventricular, surface to the outer, pial, side of the neural tube. During interphase, the cells are attached to the pial surface through endfoot structures, while during mitosis they detach from the pial side (Sauer, 1935). At later developmental stages, the CNS precursor cells become confined to the inner, ventricular zone of the brain and spinal cord. One specialized class of cells, the radial glial cells, retain the extended morphology and are anchored by endfeet at the pial side. Radial glial cells play an important role in directing the migration of differentiating neurons to the outer layers (Rakic, 1971).

It seems reasonable to surmise that the cell cycle-dependent changes in attachment and cell shape of CNS precursor cells are, at least in part, a consequence of alterations in the cytoskeleton of these cells. The composition of the IF network is cell-type specific. In the developing CNS, nestin is expressed concurrently with vimentin from neural tube closure until the end of gliogenesis. During embryonic development, the nestin protein first appears in the endfeet that contact the basal lamina on the pial surface of the neural tube, but soon nestin-containing filaments span the cell from ventricle to pia (Hockfield and McKay, 1985; Frederiksen and McKay, 1988). During terminal differentiation the IF composition rapidly switches from nestin/vimentin to \( \alpha \)-internexin and neurofilaments in neurons, and to GFAP/vimentin in astrocytes (Frederiksen and McKay, 1988; Liem, 1993; Dahlstrand et al., 1995). Nestin is nearly absent from the adult CNS (Hockfield and McKay, 1985; Lendahl et al., 1990; Zimmerman et al., 1994), with the exception of a population in the subventricular zone (Reynolds and Weiss, 1992). It is re-induced in the adult CNS in reactive astrocytes, along with vimentin and GFAP (Lin et al., 1995; Frisén et al., 1995; Galou et al., 1996; Holmin et al., 1997). Outside the CNS, nestin and vimentin are present in the somitic myotome and developing myoblasts, later followed by desmin (Lendahl et al., 1990; Valtz et al., 1991; Sejersen and Lendahl, 1993; Kachinsky et al., 1994).

SUMMARY

Neuroepithelial and radial glial cells span between the ventricular and the pial surfaces of the neural tube and express two intermediate filaments (IFs), nestin and vimentin, which form a filamentous network throughout the length of the cells. In this report we study the polymerization characteristics of nestin and examine how mutations affect the assembly and localization of the nestin protein in cultured cells and in the developing CNS of transgenic mice. A wild-type rat nestin gene transfected into the IF-free SW13 cell line failed to assemble into a filamentous network but was incorporated into the existing IF network of a subclone expressing vimentin, demonstrating that nestin requires vimentin for proper assembly. In transgenic mice, rat nestin formed a network indistinguishable from that formed by endogenous nestin and vimentin, but a mutant form lacking five amino acids at the carboxy terminus of the rod domain was largely restricted to the pial endfeet. Since nestin mRNA is localized to the pial endfoot region we propose that both transgenes are translated there, but that the wild-type protein is preferentially incorporated into the IF network. These observations provide evidence for hierarchical assembly and a complex organization of the IF network along the ventricular-pial axis in the early CNS.

Key words: Intermediate filament, Cytoskeleton, Neural tube, Radial glial cell, CNS precursor cell
Shea et al., 1993; Weitzer et al., 1995), while manipulation of IF genes in transgenic mice and analysis of hereditary diseases has provided further information about IF function. Mutations in epidermal keratins cause dominant hereditary skin blistering diseases in humans and transgenic mice (Fuchs, 1994), and targeting of the desmin gene results in cardiovascular lesions and skeletal myopathy (Li et al., 1996; Milner et al., 1996). Overexpression of normal NF subunits causes neuropathy similar to amyotrophic lateral sclerosis (ALS) in transgenic mice (Côté et al., 1993; Xu et al., 1993), while depletion of NFs slows axonal transport (Ohara et al., 1993; Eyer and Peterson, 1994). In contrast, the vimentin and GFAP genes have been demonstrated to be dispensable individually for normal development and brain function following gene targeting in mice (Colluci-Guyon et al., 1994; Gomi et al., 1995; Pekny et al., 1995).

All IFs contain an approximately 300-amino-acid residue α-helical domain, referred to as the rod domain, which is essential for polymerization (Fuchs, 1994; Liem, 1993; Steinert, 1993; Stewart, 1993). Some IFs, including vimentin, are capable of self-assembly in the absence of other IFs. In contrast, keratins and neurofilaments require two classes of subunits for assembly. Although nestin forms an IF network indistinguishable from that of vimentin and desmin (Lendahl et al., 1990), it is not yet known whether nestin is capable of self-assembly. To learn more about the interactions among intermediate filaments during early CNS development we here investigate the capacity of nestin to self-assembly and its possible requirement for vimentin. We also introduce specific mutations into the rod domain of nestin to analyze the effects on assembly and subcellular localization in CNS precursor cells of transgenic mice.

**MATERIALS AND METHODS**

**DNA constructs**

The rat nestin genomic sequence was reconstructed from Charon4A and λgt1 clones isolated in the cloning and sequencing of nestin (Lendahl et al., 1990). pNstwt was generated by deletion between two NcoI sites at 2,013 and 4,953 bases upstream of the nestin cap site in the plasmid pNc0/NesPlacZ3/3introns (Zimmerman et al., 1994). In the constructs pNstwt and pNstΔ305-309, 470 bases from −4,953 to −5,423 were also present upstream of the promoter. The primer CTCCCCACACGGACTAATCCTCGGTTGCAG was used to make a 5-amino-acid (aa) deletion in the rod 2B terminus (Mutagene kit, Bio-Rad) to generate pNst Δ305-309 (Fig. 1C). For the amino-terminal deletion construct, pNstA15, the natural ATG of nestin was replaced with the synthetic Shine-Delgarno-Kozak sequence and ATG from Nes/P3introns (Zimmerman et al., 1994). A deletion of 345 bases from the coding sequence (115 amino acids) was made by fusing the Xhol site at +427 bp from the cap site in frame to a XhoI linker placed immediately downstream of the artificial ATG. The first three aa of the truncated protein are therefore MAR. At the 3′ end the cDNA was cleaved at the NheI site and the SV40 poly(A) signal was introduced (Fig. 1D).

All the nestin promoter constructs (pNstwtIA, pNstΔ305-309HA and pNstΔ115HA) were tagged with a peptide tag recognized by an antibody to influenza virus hemagglutinin (HA) (Kolodziej and Young, 1991) in order to distinguish between the construct and the endogenous nestin protein. A complementary pair of synthetic oligonucleotides (coding sequence GGGGTAACCCCTACGACGTCCCGACTACGCCC) encoding the epitope GYPYDVPDYAP was ligated into the SmaI site at aa 1,717 in a region poorly conserved between rat and human nestin (Fig. 1B, triangle).

A non-neuronal promoter from cytomegalovirus (CMV) was used for expression of nestin in cultured cells. The coding sequence for pCMV-NeswtHA and pCMV-NstΔ305-309HA is identical to pNeswtHA and pNesΔ305-309HA, respectively, except that the CMV constructs lack the HA flag. Nestin genomic sequences from the MaeIII site at +25 in the cDNA to the NheI site below the stop codon were ligated into the NotI site of pCMV-β-gal (Clontech).

**Cell culture**

SW13 cells were the gift of Dr P. Pentchev and were grown in DMEM/F12 (Gibco/BRL) with 10% FCS (Sigma). NSELacZHipP2-21 cells were the gift of Dr Timothy Hayes and were grown in DMEM (Gibco/BRL) with 10% FCS. Cells were transfected by the method of Chen and Okayama (1987). In stable lines pSV2neo and pY3 were used for G418 resistance (0.5 mg/ml) and hygromycin resistance (0.3 mg/ml), respectively.

**Transgenic animals**

Egg donors, stud males (C57BL/6 × C3H F1), recipient females and vasectomized males (ICR) were obtained from Bommen, Denmark or from NCI, Frederick, MD. Egg donors were superovulated, mated and eggs were harvested as described (Hogan, 1986). CsCl-purified DNA was cut and fragments were separated on a 10%–40% sucrose gradient and injected in 10 nM Tris-HCl, pH 7.5, 0.1 mM EDTA at 2.5 ng/µl (Mann and McMahon, 1993).

**PCR for transgenes**

Tails or yolk sacs were digested in Proteinase K overnight according to the protocol of Laird et al. (1991). DNA was subjected to 25 cycles of 1 minute each at 94°C, 60°C and 72°C. The primer sequences for genomic rat nestin were: gNesp: GGAAGGCAA TGGGTTGTGTG; gNesup: ACAAGAGACAGCAGAGG. Reactions were carried out in Taq polymerase buffer with 2.5 mM MgCl2, 1 mM primers, 20 µM dNTPs with Taq polymerase (Perkin-Elmer).

**Sectioning and immunofluorescence**

E11.5–E12.5 embryos were collected from pregnant recipient mice or from females bred to a founder male. Embryos were fixed overnight in 4% paraformaldehyde in PBS, pH 7.2, sunk in 10% sucrose/PBS, and the trunks embedded in OCT. For vimentin staining, unfixed embryos were frozen in OCT on dry ice and sectioned, permeated with 20% polyethylene glycol 8000 and fixed in −20°C methanol. Cell monolayers were fixed in 4% paraformaldehyde in PBS, pH 7.2. Primary antibody for nestin was AS 130, diluted 1:1,000 (Tohyama et al., 1992). Monoclonal antibody V9 (Boehringer-Mannheim) was used against rat or human vimentin in cultured cells. A rabbit anti-vimentin antiserum (Moscinski and Evans, 1987) was used at a dilution of 1:80 on methanol post-fixed sections. The HA epitope tag was recognized by the monoclonal antibody 12CA5 at a dilution of 1:1,000 or 16B12 at a dilution of 1:20,000 (BABCO). Secondary antibodies were LRSC-rhodamine donkey anti-rabbit and fluorescein-goat anti-mouse (Jackson).

**Epifluorescence and confocal microscopy**

Cells were examined and photographed on a Zeiss Axioplan microscope with standard epifluorescence optics. Tissue sections were examined on a BioRad confocal microscope at ×40 magnification. A series of four optical sections at intervals of 1.98 µm were taken and projected onto a single image.

**Western blotting**

Cells were lysed in 6 M urea, 1% SDS and 50 mM Tris-HCl, pH 6.8. Laemmli gels (6%) were run and transferred to nitrocellulose (Hybond-ECL, Amersham) membranes using Towbin buffer without methanol (Harlow and Lane, 1988). AS130 was diluted 1:5,000.
Vimentin antibody V9 was used at 1:100. Appropriate HRP-conjugated secondary antibodies (Amersham) were used at recommended dilutions. Detection was carried out by chemiluminescence (DuPont/NEN). For protein extractions, cytoskeletal preparations were made by the method of Ching and Liem (1993). Cells were incubated in Triton X-100 in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, with 0.5 mM freshly added PMSF, 10 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 20 μg/ml bestatin and 20 μg/ml 3,4 dichloroisocoumarin, and centrifuged at 100,000 g for 1 hour, then treated with DNase and washed in Triton-free buffer at high speed.

RESULTS

Nestin constructs

The rod domains of all IFs are flanked by an N-terminal head domain and a C-terminal tail domain (Steinert and Roop, 1988; Fuchs, 1994). The rod domain comprises four helical segments (1A, 1B, 2A and 2B) separated by three proline- and glycine-rich linker domains (Fig. 1A). Mutational analysis has identified conserved sequences within the rod and carboxyl borders of coils 1A and 2B, respectively, that are essential for normal assembly (Fuchs, 1994). Mutations in the amino or carboxyl rod-end sequences form subunits that dominantly disrupt assembly of normal IF subunits and result in the accumulation of IF aggregates in the cytoplasm. In particular, mutations in the last 5 amino acid residues of the rod domain affect assembly capacity and inter-filament interactions for other IFs (Hatzfeld and Weber, 1992; Letai et al., 1992; Kouklis et al., 1992).

To analyse the polymerization characteristics of nestin we generated wild-type and rod-end deletion rat nestin constructs for introduction to cultured cells and transgenic mice. The principal features of the constructs are diagrammed in Fig. 1B-D. To express nestin in cultured non-neuronal cells, the genomic sequences were recloned under the CMV promoter (Fig. 1B). pCMV-NstΔ305-309 encodes a nestin protein that lacks 5 amino acid residues (LLEAE; aa 305-309) in the carboxy terminus of the rod domain.

For the transgenic experiments, constructs using the endogenous nestin promoter and enhancer elements present in the first two introns (Zimmerman et al., 1994) were made encoding wild-type nestin (pNstwtHA) and the 5-aa deletion (pNstΔ305-309HA) (Fig. 1C). To identify the encoded transgenic proteins in a background of endogenous nestin, an immunotag (hemagglutinin; HA) (Kolodziej and Young, 1991) was placed in a region of the tail domain that is poorly conserved (Dahlstrand et al., 1992). The immunotag did not alter the assembly characteristics of nestin (data not shown). A second mutation removing all of coil 1A and 61 bases of coil 1B was expressed from the nestin promoter genomic construct pNstΔ115 (Fig. 1D). This large deletion, which affects the amino-terminal domain of the rod region, was analogous to the largest deletions made in NFL and NFM (Gill et al., 1990; Wong and Cleveland, 1990).

Expression and assembly of nestin in cells containing various combinations of IFs

To study the behavior of nestin in a cellular environment devoid of IFs we introduced the wild-type rat nestin construct into SW13 cells that lack all IFs (Hedberg and Chen, 1986; Sarria et al., 1990). In vimentin-negative SW13 cells transiently transfected with pCMV-Nstwt, anti-nestin staining was evenly distributed throughout the cytoplasm (Fig. 2A). In a few cells intense spots of stain were collected on the surfaces of cells (Fig. 2B, arrows). However, filaments were never seen. Double labeling with anti-vimentin antibody demonstrated the absence of vimentin (Fig. 2C,D). These data suggest that nestin remains in a soluble, unpolymerized form in the SW13 cells. To test this hypothesis biochemically, we generated a stable cell line expressing the pCMV-Nstwt construct in the absence of vimentin. Triton X-100 extractions of this line demonstrated that nestin partitioned entirely to the soluble fraction (Fig. 2E), which is not the case...
for other assembled IFs (Lee et al., 1993; Ching and Liem, 1993).

Vimentin-positive revertants of the SW13 cell line appear spontaneously and a vimentin-positive subclone (Sarria et al., 1990) was used to study nestin assembly in the presence of an existing vimentin network. Wild-type nestin, produced from the pCMV-Nstwt construct, was incorporated into filaments in most of the cells in which it was expressed. Double immunofluorescent labeling showed that the nestin network completely coincided with vimentin filaments (Fig. 3A,B). In a small fraction of the cells, there was very strong diffuse nestin stain (Fig. 3C,D), while the vimentin-positive filaments in these cells were intact. A selected line expressing pCMV-Nstwt showed a phenotype similar to the cells shown in Fig. 3A,B and did not change over time (data not shown). These results demonstrate that nestin requires the presence of vimentin for assembly into filaments.

We next tested whether a mutant nestin protein lacking 5 amino acids from coil 2B expressed from pCMV-Nst Δ305-309 would interfere with the endogenous vimentin network. NSTΔ305-309 protein was distributed in a spotted pattern along the length of the vimentin filaments. In some cells it appeared that the vimentin network was somewhat retracted from the periphery of the cells compared to nestin (Fig. 4A,B).
1955 Analysis of nestin in transgenic mice

In selected stable transfectants expressing pCMV-NstΔ305-309 (Fig. 4C,D), nestin was distributed further toward the plasma membrane than vimentin. At the periphery, nestin remained in short filamentous structures that contained variable amounts of vimentin (arrows). Cells from the same line which did not express nestin maintained dense vimentin filaments. After prolonged culture, the abnormal appearance of the IF network intensified (Fig. 4E,F). Nestin was present in spots at the periphery of the cells while vimentin was radically withdrawn from the cell periphery, although a filamentous staining pattern persisted in the perinuclear region of the cells. These data indicate that nestin lacking 5 amino acid residues in coil 2B assembles aberrantly into the existing IF network, but that this mutant does not cause a complete dominant collapse of the vimentin network. The peripheral IF network appears to be more vulnerable than the perinuclear IF filaments to the effect of NSTΔ305-309.

Transfection of the pNstΔ115 construct into vimentin-positive SW13 cells demonstrated that this protein was rendered assembly-incompetent by the deletion of coil 1A (Fig. 4G). Anti-nestin immunoreactivity was diffuse, similar to the distribution seen for wild-type nestin in vimentin negative cells. Vimentin filaments were not perturbed (Fig. 4H).

We then asked whether excess or mutated nestin would cause disassembly of an endogenous nestin/vimentin network. The cell line NSElacZHipP2-21, derived from postnatal day 2

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**Fig. 4.** (A-F) SW13 cells expressing coil 2B deletion construct from pCMV-NstΔ305-309. (G,H) Transiently transfected SW13 cell expressing pNstΔ115HA. (A,B) Transient transfectants show differences in nestin (a) and vimentin (b) distribution. Nestin is distributed along vimentin filaments but is found in small discrete particles. Areas of the cell periphery are stained with nestin but not with vimentin, as indicated by arrows. (C,D) Nestin aggregates are more visible at the periphery of the cells in a stable, selected cell line transfected with pCMV-NstΔ305-309 after four weeks in culture. Vimentin staining was retracted from peripheral regions of the cells in D. (E,F) Nestin-mediated disruption of vimentin distribution was pronounced after seven weeks in culture. In E, nestin is present in filaments which remain perinuclear, but do not appear to be aggregated (compare to Fig. 3E). Vimentin intermediate filaments are confined to a perinuclear area and coincide with nestin filaments in this region. Vimentin staining is absent at the cell periphery. (G) Cells 96 hours after transfection with pNstΔ115HA. Multinucleate cells are common in the parental SW13 cell line. (H) Vimentin IF distribution is normal in these cells. Bars: 4.75 μm (A-F); 20 μm (G,H).
(P2) hippocampus expresses both vimentin and nestin (T. Hayes, unpublished results). In cells transfected with the wild-type construct pNstwtHA, distribution of total and introduced HA-tagged rat nestin coincided (Fig. 5A,B). The distribution of the nestin protein encoded by pNstD305-309HA differed slightly from that of the endogenous or wild-type rat nestin. The coil 2B mutant nestin was distributed in a more markedly perinuclear fashion than the endogenous nestin, which appeared to be normally distributed. The mutant nestin incorporated into filaments, but immunofluorescence tapered off toward the periphery of the cell while the endogenous protein did not (Fig. 5C,D). It has been noted that microinjected vimentin is added to an IF network at the nuclear membrane (Vikstrom et al., 1989), although expression of vimentin from a plasmid resulted in assembly along existing intermediate filaments throughout the cytoplasm (Ngai et al., 1990). These results demonstrate that the mutant protein does not alter the endogenous nestin-vimentin filament network in CNS-derived cells. The presence of endogenous nestin, however, appears to limit incorporation of mutant nestin into an IF-like pattern in some, but not all, parts of the cell.

Western blot

Urea extractions on transient transfectants and on stable SW13 lines containing wild-type and mutant nestin demonstrated that the nestin protein is of normal size and shows little degradation (Fig. 6). Lanes 1 and 2 show extracts from lines expressing nestin from pCMV-NstΔ305-309. Lanes 3 and 4 compare two lines expressing pCMV-Nstwt. Lane 5 shows the parental SW13 vimentin positive line, and lane 6 the parental IF negative line. Under these extraction conditions, neither mutant nor wild-type rat nestin appears to be degraded, and the ratios of wild-type and mutant nestin to vimentin are similar.

Wild-type and mutated nestin localize differently in the developing CNS of transgenic mice

To investigate the effect of expression of wild-type and mutated nestin in vivo, we generated transgenic mice expressing the wild-type and 5 amino acid deletion constructs. Transgenic mouse embryos developing from injected zygotes and established lines were collected at E11.5 to E12.5. Nestin protein from the wild-type construct pNstwtHA was detectable in 100% of the transgenic animals examined. Expression in the pNstΔ305-309HA animals was more variable, ranging from levels similar to those seen in pNstwtHA mice to levels detectable only by the more sensitive anti-HA antibody 16B12. We could not detect any morphological, behavioral or physiological differences between transgenic mice of either strain and wild-type littermates. The transgenes were transmitted to approximately half (47%) of the offspring from heterozygous founders. However, analysis of the distribution of the two forms of nestin

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**Fig. 5.** (A,B) NSE-LacZHipP2-21 (Hip21) cells transfected with pNstwtHA expressing HA-tagged wild-type nestin. (A) Tagged nestin is stained with anti-HA antibody. (B) Endogenous and tagged nestin are stained with AS130. Both forms colocalize throughout the cells. (C,D) Hip21 cells from stable selected line expressing pNstΔ305-309HA. (C) Cells are stained for the HA flag on nestin. (D) Same cells expressing endogenous nestin. The mutant protein is not distributed as far toward the periphery of the cells as the endogenous nestin. Bar, 13.6 μm.
revealed a difference in the subcellular distribution of the transgenic proteins.

Localization of transgenic nestin protein in the embryonic spinal cord

Transgenic embryos carrying the wild-type HA-tagged construct, pNstwHA, were doubly labeled with antinestin and anti-HA antibodies to identify total nestin and transgenic nestin, respectively. The distribution of transgenic nestin (Fig. 7A) was indistinguishable from that of total nestin (Fig. 7B). The antibodies detected pial endfoot and radially arrayed processes that spanned the spinal cord from the ventricular zone to the pial surface. Staining was strongest at the pial endfoot and the portion of the process proximal to them for both antibodies, as described for Rat 401 (Hockfield and McKay, 1985), but was clearly visible throughout the radial processes in all embryos analysed, even at high dilutions of the primary antibody.

While the overall morphology of the brain and spinal cord was indistinguishable from that of non-transgenic littermates and pNstwHA mice, in transgenic embryos bearing the pNstΔ305-309HA construct, the distribution of the mutant transgenic nestin protein differed from the wild-type transgenic nestin described above. In the animals expressing the pNstΔ305-309HA construct the mutant protein localized primarily to the pial endfoot and to the filaments in their immediate vicinity (Fig. 7C). Accumulation of mutant transgenic nestin in the endfoot did not affect the distribution of total nestin (Fig. 7D). Transgenic nestin appeared to be continuous along the ventro-lateral pial surface due to the projection of several optical planes onto one image (Fig. 7E,F). Mutant nestin was excluded from central nervous system (CNS) processes in the ventral-medial portion of the spinal cord, but perinuclear staining was occasionally detected in cells in the ventricular zone. In the dorsal half of the spinal cord, anti-HA immunoreactivity was more frequently distributed along the length of the radial cells than in the ventral spinal cord. Total nestin immunoreactivity was not restricted in any part of the spinal cord (Fig. 7F). Taken together, these data suggest that the transgenic nestin protein is predominantly localized to the pial endfoot region. It could be argued that this is a consequence of lower expression of the mutant transgenic protein. In contrast to the NstwHA embryos, which expressed detectable HA-tagged protein in 100% of the embryos tested (6/6), only 8 of 17 of the NstΔ305-309HA transgenic embryos expressed detectable levels using the less sensitive antibody 12CA5, although HA-tagged protein was detected in 8/8 embryos re-examined using the more sensitive monoclonal anti-HA antibody 16B12. This explanation, however, seems unlikely, because the wild-type rat nestin was consistently detected in both endfoot and medial processes, even at very high dilutions of the primary antibody.

Analysis of vimentin distribution in both non-transgenic and pNstΔ305-309HA animals shows that the pattern of vimentin immunoreactivity is not altered in the animals bearing the mutant construct. In Fig. 8A a non-transgenic littermate displays a normal vimentin pattern and in Fig. 8B, a pNstΔ305-309HA transgenic animal shows a similar even distribution across the width of the neural tube, without high concentration in any endfoot structures. This result shows that although the mutant nestin is restricted to the pial region of the neural tube, it does not cause a redistribution of the vimentin network.

DISCUSSION

The data presented in this study provide new information about the assembly characteristics and subcellular localization of IFs during early CNS development. First, nestin requires vimentin in order to assemble into an IF-like network in cultured cells. Second, deletions in the rod domain affect assembly in cultured cells and alter subcellular protein localization in CNS precursor cells and radial glial cells in vivo. Third, the nestin rod C terminus mutation is not a dominant mutation, in contrast to similar mutations in other IFs. Fourth, the data suggest that the most important site of translation of nestin in CNS precursor cells is the pial endfoot.

Wild-type nestin requires vimentin for assembly

Introduction of wild-type nestin into a IF negative cell line demonstrated that nestin is incapable of self-assembly, but in the presence of vimentin, nestin co-assembled into the IF network. The inability to self-assemble is consistent with the very short (7 aa) head region of the nestin protein (Lendahl et...
Sequences in the head domain of desmin and vimentin are essential for self-assembly capacity (Herrmann et al., 1992; Hoffmann and Herrmann, 1992). Furthermore, a headless vimentin is soluble and can assemble with wild-type subunits only if it comprises less than one fourth of the tetramer components (Andreoli and Trevor, 1994).

Nestin thus joins the neurofilaments and the keratins among the obligate heteropolymer IF subunits. This result points to an interesting conservation of assembly characteristics among the IF members expressed throughout neuronal differentiation. During early CNS development, the large IF nestin is dependent on the presence of the smaller vimentin for assembly, while at later stages the two larger forms of neurofilaments are dependent on the smaller \( \alpha \)-internexin or NF-L chain for assembly. This similarity between early and late stages becomes more conspicuous, considering that nestin and neurofilaments are closely related in evolution (Dahlstrand et al., 1992; Weber et al., 1991), and both nestin and the neurofilament heavy chain contain very long, repetitive carboxy-terminal tail regions. The dependence of nestin on vimentin may also explain why the two IFs are nearly always coexpressed, both during early CNS development and during early myogenesis (Sejersen and Lendahl, 1993; Sjöberg et al., 1994).

A coil 1A and 1B mutation is more severe than a small coil 2B deletion in cultured cells

Transfection of pNst\( \Delta \)115, which codes for nestin lacking coils 1A and 1B, showed that this construct was assembly-incompetent, regardless of the IF environment. This finding is consistent with the observations of similar mutations in desmin, neurofilaments and keratins (Albers and Fuchs, 1989; Gill et al., 1990; Raats et al., 1990; Wong and Cleveland, 1990; Chin et al., 1991). For keratin IFs, the coil 1A sequence is essential for the earliest stages of assembly with other subunits (Coulombe and Fuchs, 1990).

Unexpectedly, the coil 2B mutation in nestin did not affect polymerization of endogenous vimentin and nestin, and affected vimentin assembly only mildly in nestin-free cells. The pNst\( \Delta \)305-309HA protein became partially incorporated into networks in cells expressing vimentin, although in an abnormal, discontinuous fashion. Despite this incorporation it did not alter vimentin polymerization, except that vimentin filaments were not present at the periphery of the cells. With endogenous vimentin and nestin present the mutant protein appeared to assemble normally in parts of the cell and did not result in filament collapse.

The conserved IF consensus region of the carboxyl terminus of the coil 2B rod domain (in nestin, EVATYRTTLLEAE) has been demonstrated to play an important role in the assembly and morphology of other intermediate filaments. Deletions and point mutations of the last 4-10 aa of the conserved rod end domain in desmin, NF-M, NF-L and keratin 14 result in dominant collapse of the entire cytoplasmic IF network (Heins et al., 1993; Raats et al., 1992; Coulombe et al., 1990; Wong and Cleveland, 1990; Gill et al., 1990; Albers and Fuchs, 1987; van den Heuvel et al., 1987). Point mutations in keratin...
subunits cause several blistering diseases of the skin and the rod C-terminal consensus sequence is one of the hot spots for these mutations in epidermal keratins (Fuchs, 1994, and references therein). In vitro experiments demonstrated that the (R/K)LLEG sequence is required for lateral alignment of keratin filaments (Wilson et al., 1992). In contrast, pCMV-NstΔ305-309 caused only a subtle redistribution of filaments and retraction from the periphery of the cells. These observations suggest that there may be a difference in the capacity of nestin to integrate into IFs, since comparable mutations cause greater disruption in IFs of different composition. Indeed, a greater proportion of nestin is soluble compared to vimentin (Almazan et al., 1993).

The coil 2B mutation alters the localization of nestin in transgenic mice

When wild-type rat nestin and the pNstΔ305-309HA nestin protein were produced in the early CNS of transgenic mice the two proteins localized quite differently. The transgenic wild-type nestin mimicked the distribution of endogenous nestin whereas the mutant protein was confined to the pial endfeet. As in cell monolayers, the mutant protein did not alter the distribution of endogenous vimentin and nestin, which further supports the conclusion that the rod end mutation is not dominant. The lack of an overt phenotype in the transgenic animals is likely to be a reflection of the lack of change in the distribution of endogenous nestin in vivo. However, the mutant protein failed to become incorporated into filaments in transgenic animals, while in cultured cells, the mutant protein was distributed in a pattern largely overlapping that of the endogenous nestin protein. This suggests that the diffusion of the mutant protein is limited in this vivo and that the endogenous nestin protein is preferentially incorporated into filaments, a preference that is not evident in NSElacZHipP2-21 cells.

The accumulation in the endfeet of NSTΔ305-309 protein is most likely a result of a combination of mRNA sorting, localized translation and an inability to integrate into existing IF networks. Endogenous nestin mRNA is localized to the pial endfoot region (Dahlstrand et al., 1995; Zimmerman et al., 1994). This distribution appears to be specific for nestin, since RNA from vimentin (Dahlstrand et al., 1995) and LacZ expressed from the nestin promoter (Zimmerman et al., 1994) are found much closer to the ventricular side. Nestin mRNA shows a similar sorting during early muscle development, where the mRNA is confined to the region of the myotubes located closest to the myotendinous junction, while the protein is more uniformly distributed (Wroblewski et al., 1997).

Localized translation of nestin in the endfeet also receives support from the observation that the first nestin immunoreactivity in the neuroepithelial cells appears at the pial surface (Hockfield and McKay, 1985). The most likely explanation for the difference in protein distribution in the neuroepithelial cells between wild-type and pNstΔ305-309HA nestin would then be that wild-type nestin can incorporate into the existing vimentin/nestin network in the neuroepithelial cell and become distributed along the length of the cell, while this is not the case for the pNstΔ305-309HA protein, which has a reduced capacity for assembly into networks.

The data presented here give support to the view that different regions along the ventricular-pial (or proximal-distal) axis are distinct molecular environments. In addition to the distinct subcellular localization of nestin and vimentin mRNAs, there is substantial evidence that the subpial zone in the developing spinal cord, cortex and optic tectum is a molecularly distinct compartment with active mechanisms to localize proteins and mRNA preferentially. For example, GFAP immunoreactivity is first observed at the pial surface of the cell and only later becomes distributed across the radial glial cell (Yang et al., 1993). Two antibodies to chick optic tectum recognize antigens that become restricted to the pial endfeet and superficial processes during development of radial glia (Herman et al., 1993). The differential localization of these proteins strongly suggests that there is a mechanism for the distribution of particular proteins to the pial surface of the cell.

The composition of the extracellular matrix differs on the ventricular and pial surfaces (Thomas and Dziadek, 1993). Furthermore, the positions of retinal ganglion cells bodies and axons can be reversed in the eye by treatment with chondroitin sulfate (Brittis and Silver, 1994).

A prediction of our model is that the final vimentin/nestin network in the neuroepithelial cells is a result of assembly of the two proteins initially localized to opposite ends of the cell and that protein transport in the cell may be required to integrate the IF subunits into the network. We can only speculate about the biological significance of this initial polarization, but it may be that it endows the cell with different ratios of vimentin and nestin in different regions, i.e. higher nestin in the endfoot region. Such a difference in cytoskeletal composition may be linked to the observation that the pial endfeet undergo a cycle of attachment/detachment during the cell cycle. A number of IF-associated proteins linking an IF
network to desmosome- and hemidesmosome-like structures at the cell surface have been identified, e.g. desmoplakin, BPAG1, plectin and IFAP300 (Chou et al., 1997). It is possible that these or related proteins, together with nestin and/or vimentin, organize the attachment/detachment at pial endfeet during the cell cycle of the proliferating CNS precursor cells. Evidence for dynamic behavior of vimentin and nestin during the cell cycle comes from phosphorylation studies. Vimentin IFs undergo cycles of phosphorylation and depolymerization at every cell division (Chou et al., 1990), and treatment of oligodendrocyte precursors with a phosphatase inhibitor partitioned more nestin to the soluble pool (Almazan et al., 1993). Taken together, the data presented here add to an emerging picture of differentially localized ECM, ECM-interacting and cytoskeletal proteins in the neuroepithelial cells, and provide evidence for complex sorting and hierarchical assembly processes of the IFs in these cells.

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