INTRODUCTION

A cellular differentiation process in which centriologenesis takes place according to the acentriolar pathway occurs in epithelial cells from reproductive and respiratory tracts in vertebrates (for a review see Tournier and Bornens, 1994). The lining tissue of the upper respiratory tract is a pseudostratified epithelium composed of three main cell types, basal cells, secretory cells and ciliated cells. This epithelium is the first target for a wide range of inhaled molecules and particles, and mucociliary clearance is responsible for their partial elimination: beating of cilia along the upper respiratory tract continuously drives the mucus towards nasopharynx. Most of the molecular events underlying the ciliated cell differentiation process remain to be understood while detailed ultrastructural features have been described in different species (for a review see Dirksen, 1991). Indeed, the formation of approximately 200 cilia in terminally differentiated ciliated cells requires the assembly of the same number of centriole/basal bodies. Procentrioles are assembled around electron-dense cytoplasmic granules near the Golgi area. The centrioles elongate, migrate in the cytoplasm and anchor to the apical plasma membrane where they trigger axonemal microtubule polymerisation and cilia formation (Sorokin, 1968; Lemullos et al., 1988).

Development of mammalian primary cultures of respiratory epithelial cells has allowed the analysis of the expression of differentiation markers during in vitro mucociliary differentiation. Differentiation can be triggered using air-liquid interface culture conditions (Clark et al., 1995; Tournier et al., 1998; LeDizet et al., 1998), suggesting that similar cellular constraints regulate differentiation both in vitro and in vivo. Another experimental system allowing mucociliary differentiation has been reported for human cells: cells dissociated from nasal polyps (Jorissen et al., 1991; Million et al., 1999) or from nasopharynx (Boxberger et al., 1993) are seeded on thick type I collagen matrix. Epithelial sheets obtained after 2 or 3 weeks of cell proliferation are then able to differentiate into ciliated cells as cell spheroids.

Centrin proteins are centrosomal components which are associated with centrioles (Baron et al., 1992; Paoletti et al., 1996) and which belong to the Ca2+-binding protein family. First identified in the striated flagellar roots of the green algae *Tetraselmis striata* (Salisbury et al., 1984), centrin has been

SUMMARY

Differential expression and cellular distribution of centrin isoforms during human ciliated cell differentiation in vitro

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Centrin protein is an ubiquitously expressed cytoskeletal component and is a member of the EF-hand superfamilly of calcium-binding proteins. It was first discovered in the flagellar apparatus of unicellular green algae where it is involved in contraction of Ca2+-sensitive structures. Centrin protein is associated with centrosome-related structures such as spindle pole body in yeast, and centriole/basal bodies in flagellar and ciliated cells. Three centrin genes have been cloned in human cells. In this work, we have performed a comparative biochemical and functional analysis of centrin isoforms using a primary culture of human nasal epithelial cells which provides an efficient way to obtain a complete ciliated cell differentiation process. RT-PCR experiments show that the expression of the three human centrin genes increases during cell differentiation, and that only centrin 2 and 3 are expressed during cell proliferation. Using polyclonal antibodies raised against recombinant human centrin 2 and 3, we show a specific pattern of protein expression. Ultrastructural immunolocalization suggests that centrin proteins are involved in the early process of centriole assembly, as they are concentrated within the precursor structures of centriole/basal bodies. It also shows a differential localisation of centrin proteins in mature centriole/basal bodies, suggesting different functions for centrins 1/2 and centrin 3. This is also supported by functional analyses showing that centrin 1 and/or centrin 2 are involved in ciliary beating.

Key words: Centrin, Human respiratory epithelial cell, Differentiation, Ciliogenesis, Centriole biogenesis

INTRODUCTION
characterised in a variety of eucaryotes (for review see Salisbury, 1995; Schiebel and Bornens, 1995). Using anticientrin antibodies in protozoa, centrin was shown to be associated with microtubule organising centres or accessory structures (Levy et al., 1996; Klotz et al., 1997). In contrast, in ciliated epithelial cells, centrin is located within the distal lumen of centriole/basal bodies (Levy et al., 1996; LeDizet et al., 1998). Three centrin genes have been identified so far in human cells (Errabolu et al., 1994; Lee and Huang, 1993; Middendorp et al., 1997). The different centrin isoforms could not be distinguished using polyclonal and monoclonal antibodies raised against centrin from unicellular green algae (Sanders and Salisbury, 1994; Paoletti et al., 1996). Only RT-PCR experiments using specific primers have allowed a comparative study of centrin gene expression (Wolfrum and Salisbury, 1998; Le Dizet et al., 1998). Centrin 2 was shown to be ubiquitously expressed while centrin 1 was restricted to retina and testis. A differential regulation of the three human centrin genes suggested specific functions. In Saccharomyces cerevisiae, Cdc31p, which is highly related to HsCen3p (Middendorp et al., 1997), is implicated in SPB duplication (Baum et al., 1986; Spang et al., 1993). On the other hand, centrin from the green alga Chlamydomonas reinhardtii, which is more related to centrin 1/2, forms Ca2+-dependent contractile fibres which are required for the proper segregation of the flagellar apparatus during cell division (Kuchka and Jarvik, 1982; Wright et al., 1985). Moreover, flagellar excision in this algae has been shown to be mediated by a contraction of centrin-based stellate fibres of the transition zone (Sanders and Salisbury, 1994). Centrin isoforms are thus candidates for several functions either in centriole assembly or in their segregation in both centriolar and acentriolar pathways, i.e. centriole assembly during centrosome duplication or during ciliated cell differentiation, respectively.

In this work, we show that the expression of the three centrin genes increased during mucociliary differentiation of human nasal epithelial cells. HsCEN1 mRNA was not detected by RT-PCR during epithelial cell proliferation while HsCEN2 and HsCEN3 were expressed. Using two polyclonal antibodies raised against recombinant human centrin 2 and 3, we show a specific pattern of protein expression and a differential localisation of centrin isoforms in centriole/basal bodies. Centrin isoforms are concentrated within the cytoplasmic precursors of centriole/basal bodies, suggesting a role in the early steps of centriole assembly. Finally, in correlation with their differential localisation in the centriole/basal bodies and the transition zone of the axonemes, our results suggest that HsCen1/2p is involved in the regulation of ciliary beating.

### MATERIALS AND METHODS

**Primary cultures of human nasal epithelial cells**

Nasal polyps obtained from patients suffering from nasal polyposis or turbinate tissues obtained after turbinectomy from healthy volunteers were obtained from Hospital Intercommunal de Créteil (Dr A. Coste, Service de Pr Penyengre).

Explants from human nasal polyps or turbinate tissues were preplated for 1-2 hours at 37°C on plastic dishes (Falcon) to allow the formation of cell spheroids. At C+2 days, UG was replaced by 10% NuSerum (NuS, Beckton Dickinson). In these conditions, epithelial cell spheroids differentiated into secretory and ciliated cells.

**Antibodies**

Three polyclonal antibodies (C1, C2, C3) were raised against recombinant human centrins 1, 2 and 3, respectively. C3 was shown to be specific for HsCen3p while C1 and C2 both recognised HsCen1p and HsCen2p. Anti-centrin polyclonal Abs (C1, C2, C3) were affinity purified as described in Tassin et al. (1998). DM1A, a monoclonal antibody against α-tubulin was purchased from Sigma. GT35, a monoclonal antibody directed against glial glutamylated tubulin was prepared using a glutamylated peptide as immunogen (Wolf et al., 1992). CTR910 is a mAb obtained after immunisation of mouse with a fraction enriched in human lymphoid centrosomes.

**Immunofluorescence**

Epithelial spheroids or epithelial outgrowths were washed in PHEM (45 mM Pipes, 45 mM Hepes, 10 mM EGTA, 5 mM MgCl2, and 1 mM PMSF, pH 6.8) buffer and fixed in −20°C methanol, or pre-extracted in PHEM buffer containing 0.05% Triton X-100 for 30 seconds, rinsed in PHEM buffer and fixed in −20°C methanol. Alternatively, cells were fixed in 3% paraformaldehyde (PFA) for 1 hour and post-fixed in methanol. In all cases, immunofluorescence was performed as described by Paoletti et al. (1996). At the end, spheroids or coverslips were washed in PBS, dehydrated in ethanol and mounted in Citifluor for confocal microscopy analyses.

**RT-PCR analysis**

Total cellular RNAs were prepared according to the method described by Chomczynski and Mackey (1995). cDNA was synthesised from 10-20 μg of total RNA in a 20 μl volume reaction mixture containing 10 μCi μl−1 [α-35P]dCTP, oligoT primer and 200 units of SuperScript II reverse transcriptase (Gibco-BRL). cDNA fragments were purified using Biogel P10, quantified, diluted and used as templates at 1 ng for PCR. The following pairs of forward and reverse primer sets were used for amplification:

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>centrin 1</td>
<td>5'-GTG-AAA-CCT-TAG-AGG-ACA-GC(G)-3'</td>
<td>5'-AGT-GGA-CAG-GCT-AGG-CAA-CT(I)-3'</td>
</tr>
<tr>
<td>centrin 2</td>
<td>5'-TCG-GAT-CAT-GAA-GAA-GAC-CA(G)-3'</td>
<td>5'-CCA-AAG-AAG-TGA-CAA-AGG-GAA-(C)-3'</td>
</tr>
<tr>
<td>centrin 3</td>
<td>5'-AAC-GGC-TAA-AAT-AGG-CTC-AA(G)-3'</td>
<td>5'-GAA-GGG-ACA-GGA-AAA-ATG-AA(C)-3'</td>
</tr>
<tr>
<td>centrin 4</td>
<td>5'-GAG-AAC-TGT-CTG-AGG-AAC-AG(A)-3'</td>
<td>5'-TCA-TAG-TGA-CTA-ACT-CTT-(C)-3'</td>
</tr>
</tbody>
</table>

All PCR amplifications were performed with a Perkin Elmer apparatus using long expand Taq polymerase (Boehringer) with 10% DMSO. Products were run on 2% agarose electrophoresis gels and stained with ethidium bromide. PCR fragments were sequenced using...
Protein analysis

Cells dissociated from nasal polyps or turbinates (day 0) or from epithelial spheroids during mucociliary differentiation were directly resuspended in SDS-PAGE sample buffer or in IEF sample buffer for 2-D electrophoresis (total protein extracts). Alternatively, cells were lysed in 0.5% Triton X-100 in PHEM buffer containing protease inhibitors. Proteins were fractionated into pellet (insoluble) and supernatant (soluble) by centrifugation at 10,000 g for 20 minutes. ‘Soluble’ and ‘insoluble’ proteins were recovered in SDS-PAGE sample buffer or in IEF sample buffer.

SDS-PAGE was performed according to the method of Laemmli (1970) on 15% polyacrylamide gels. For 2-D gel electrophoresis, isoelectric focusing was performed on a mixture of pH 4-6.5 and pH 3.5-10 (4/1) ampholines according to O’Farrell (1975). Electrophoresis was performed in 0.9 mm capillary tubes for 16 hours at 400 V. The second dimension analysis (SDS-PAGE) was carried out on 12.5% polyacrylamide gels. After transfer onto a nitrocellulose filter, polypeptides were fixed according to the protocol of Van Eldik and Wolchok (1984) by incubation with 0.2% glutaraldehyde in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 15 minutes at room temperature. Immunodetection was performed as described by Paolletti et al., (1996) except that a secondary antibody coupled to peroxidase and ECL revelation was used.

Immunogold electron microscopy

Epithelial spheroids were rinsed in PBS and fixed in 3.7% paraformaldehyde, 0.2% glutaraldehyde in PBS for 60-90 minutes. After centrifugation (300 g, 5 minutes), they were rinsed 3 times in PBS and once in 0.1 M glycine in PBS. Pellets were overlaid with 3% gelose to recover cellular aggregates. Spheroids were dehydrated in a graded ethanol series, embedded in LR-White and polymerised for 72 hours at 45-50°C. Sections (70 nm) were placed onto nickel or gold grids and used for the post embedding immunolocalization of centrin antigens. After a preincubation step in 0.1 M glycine, 2.5% BSA in PBS for 20 minutes, LR-White sections were incubated with the primary antibody (C1, C2, or C3 at 1:100) in PBS containing 0.5% BSA, 0.05% Tween-20 overnight at 4°C and/or for 1 hour at room temperature. Gold (10 nm) coupled Protein A (1:50) was incubated for 90 minutes at room temperature and samples were post fixed with 2.5% glutaraldehyde in PBS. All washes were performed in PBS. After double contrast with uranyl acetate and lead citrate, sections were observed with a JEOL (JEM100C) electron microscope.

Effect of anti-centrin antibodies on ciliary beat frequency

The method described by Clary-Meinesz et al. (1992) was adapted for ciliated epithelial spheroids differentiated in vitro. A spheroid was put on a microscope slide at room temperature (20°C) and held in position with a micromanipulator (Leitz, type M). Culture medium was on a microscope slide at room temperature (20°C) and held in position with a micromanipulator (Leitz, type M). Culture medium was on a microscope slide at room temperature (20°C) and held in position with a micromanipulator (Leitz, type M). This method allowed the observation of ciliary beating directly on a video camera (Sony, CCD-Iris) connected to a VHS video tape recorder (Sony). Image analysis was performed using an automated computer-assisted system, described by Nasr et al. (1995), based on variation of grey levels caused by cilia movement. For each measurement, 5 different areas were selected, each area localised on the apex of a single ciliated cell. Results were given as mean ± s.d. and compared using Student’s t-test or Mann-Whitney’s non parametric U test when variances’ homeoscedasticity was not verified.

In parallel, purified lgs used in the ciliary beat assay were used at the same concentrations in an immunofluorescence study of demembranated spheroids which were fixed in 3% PFA after the antibody incubation (5 minutes). Spheroids were the incubated with GT335, rinsed in PBS and further incubated with secondary antibodies coupled to fluorescein or rhodamine. Spheroids were washed in PBS, dehydrated in ethanol and mounted in Citifluor for confocal microscopy analyses.

RESULTS

Characterisation of centrin isoforms in human nasal epithelial cells

We first tested the localisation of centrin proteins in human nasal epithelial cells in primary culture using the explant technique and two polyclonal antibodies (C2, C3) raised against recombinant human centrin 2 (HsCen2p) and 3 (HsCen3p), respectively. C3 recognised specifically HsCen3p (see below). C2 serum, however, or purified immunoglobulins, did not discriminate between human centrin 1 (HsCen1p) and HsCen2p due to the high homology of the two proteins.

Double immunofluorescence experiments with anti-α-tubulin (DM1A) (Fig. 1A, b and e) and either C2 (Fig. 1A, a) or C3 (Fig. 1A, d) showed that both C2 and C3 recognised centrosomes of non-ciliated epithelial cells. In ciliated cells, they stained punctate structures located at the basis of cilia anoxones, likely to be centriole/basal bodies. A weak staining of the cilia was also observed with C2 (see inset in Fig. 1A, a) but not with C3 (see inset in Fig. 1A, d).

In cells dissociated from human nasal polyps or turbinates, C2 detected a 20-21 kDa doublet polypeptide while a single polypeptide of higher molecular mass was detected with C3 (Fig. 1B, a). This was confirmed by the successive incubation of the nitro-cellulose membrane with C2 and C3 (Fig. 1B, a). C1, raised against recombinant human centrin 1 (HsCen1p) detected the same polypeptide doublet, corresponding to Hscen1p and Hscen2p (data not shown). Centrin isoforms were enriched in Triton X-100-insoluble protein fractions. In parallel, Triton X-100 soluble and insoluble protein fractions were separated on SDS-polyacrylamide gels containing 2 mM Ca2+ or 2 mM EGTA. In these conditions, the migration profile of centrin isoforms recognised by C2 was basically unchanged, although the two polypeptides were thus not clearly separated (Fig. 1B, b). Moreover, treatment with EGTA or Ca2+ changed the partition of soluble and insoluble centrin polypeptides. Two polypeptides were detected with C3 in both Triton X-100 soluble and insoluble fractions in the presence of Ca2+ (Fig. 1B, b), suggesting that the slow migrating isofrom corresponded to Ca2+-bound HsCen3p.

Centrin expression during proliferation and mucociliary differentiation

Cells dissociated from human nasal polyps or turbinates (day...
were cultivated on thick type I collagen gels. At confluence, epithelial sheets recovered after collagenase treatment were induced to form spheroids on a rotary shaker for 5-8 days. Epithelial spheroids were then maintained in suspension using culture plastic flasks. During the latter phase, the proportion of secretory and ciliated cells progressively increased. Epithelial cells from spheroids were dissociated during the time-course of mucociliary differentiation and the percentage of ciliated cells was estimated by flow cytometry using GT335 as a specific antibody (Tournier et al., 1998). This monoclonal antibody, raised against glutamylated tubulin peptides (Wolff et al., 1992), specifically recognised cilia axonemes and centriole/basal bodies of mammalian respiratory epithelial cells (Tournier et al., 1998; Million et al., 1999). We checked that the GT335-positive cell population in flow cytometry corresponded to ciliated cells. The percentage of ciliated cells was variable from one experiment to another. At confluence, no ciliated cells were detected, and during the first week after confluence, the percentage of ciliated cells was always low, but further increased for longer periods of culture (data not shown).

During these experiments, centrin expression was followed by RT-PCR analyses. The three centrin mRNAs were detected at day 0 (D0) and during differentiation. Centrin 1 mRNA however was not detected during epithelial proliferation (Fig. 2A, a) while centrin 2 and centrin 3 mRNAs were detected during both proliferation and differentiation (data not shown). When PCR amplification was limited to 35 cycles, we could detect a progressive increased transcription of the three centrin genes during differentiation (Fig. 2A, b).

We performed western blot analyses of total proteins and Triton X-100 soluble and insoluble enriched protein fractions using C2 and C3 sera. For an equal amount of cells, an increased amount of centrin was observed using both C2 (Fig. 2B, a) and C3 (Fig. 2B, b) sera. Centrin isoforms were progressively and preferentially enriched in Triton X-100-insoluble protein fractions (Fig. 2B, c and d). Two-dimensional electrophoretic separation of Triton X-100 insoluble and soluble proteins at day C+16 revealed a strikingly different pattern when C3 and C2 were used successively: a unique spot which corresponds to HsCen3p (Fig. 2C, a) whereas at least 6 centrin isoforms were recognised by C2 (Fig. 2C, b and b'). These experiments confirmed that C3 did not cross-react with HsCen1p and HsCen2p (Fig. 2C, b and b').
The complex pattern recognised with C2 could correspond to different isoforms of both HsCen1p and HsCen2p and/or different posttranslational modifications of the two centrin gene products.

**Differential localisation of HsCen1/2p and HsCen3p**

To localise the centrin isoforms during ciliated cell differentiation, we first carried out an IF analysis using laser confocal microscopy. Epithelial spheroids were fixed either in methanol or in paraformaldehyde during differentiation and submitted to immunofluorescence experiments. Early during the differentiation process, mAb GT335 and C2, used for double staining, decorated the centrioles of non-ciliated epithelial cells (Fig. 3, a and b). Double labelling with mAb GT335 and both anti-centrin antibodies of in vitro differentiated epithelial cells revealed that centrin proteins were present in centriole/basal bodies of ciliated cells (Fig. 3, c-f). In addition, a punctate and conspicuous cytoplasmic staining was revealed with both C2 and C3 in some non-ciliated epithelial cells (arrowheads in Fig. 3, c+d and 3, e+f), suggesting the presence of centrin proteins in precursor cytoplasmic structures for the assembly of centriole/basal bodies. This was confirmed on EM sections of epithelial spheroids during differentiation (Fig. 4). Centrin proteins as revealed by C2 (Fig. 4a) and C3 (Fig. 4a') were localised near electron-dense fibrogranular aggregates that were previously described as the first cytoplasmic structures involved in centriologenesis (Sorokin, 1968; Dirksen et al., 1991). C2 and C3 also decorated centrioles assembled around dense cytoplasmic granules and centrioles migrating in the cytoplasm (Fig. 4b and Fig. 4b', respectively). When centrioles were cut longitudinally, gold particles were shown to be enriched in their distal lumen (arrows in Fig. 4b and b'). Finally, centrin proteins were localised specifically in the lumen of centriole/basal bodies anchored at the apical plasma membrane (Fig. 4c and c'). Interestingly, localisation of HsCen3p was restricted to centriole/basal bodies (Fig. 4c') while HsCen1p/2p were also localised in the proximal part of the axonemes (Fig. 4c'). Moreover, when the relative solubilisation of centrin proteins with detergent or urea was achieved, HsCen1/2p and HsCen3p behave differently: half of HsCen1/2p was solubilized after 8 M urea treatment while HsCen3p was almost totally extracted (data not shown). Thus, part of HsCen1p or HsCen2p may correspond to ‘insoluble’ structures associated with the axoneme.

**Specific inhibition of ciliary beating by C2 immunoglobulins**

Centrin proteins belong to the Ca^{2+}-binding protein family.
and Ca\textsuperscript{2+} was shown to directly influence the regulation of ciliary beating (Brokaw, 1991). We thus tested the effect of specific anti-centrin antibodies on ciliary beating. Ciliary beat frequency was measured on ciliated epithelial spheroids differentiated in vitro. Video-recordings were analysed using a image analysis process (see Materials and Methods). Ciliary beat frequency recorded for native spheroids incubated in CBF buffer gave an average value of 6 Hz (C, Fig. 5A). After demembranation, all cilia were immobile. Incubation of demembranated epithelial spheroids in a ‘ATP-containing reactivation buffer’ reactivated the cilia to 5 to 6 Hz (R, Fig. 5A) and CBF was almost constant over a 30 minutes incubation period (Million et al., 1999). Purified Igs from C3 and C2 sera were applied in a range from 1 to 125 \( \mu g.mL^{-1} \) onto reactivated cilia. One ciliated epithelial spheroid was chosen and two successive Igs concentrations were applied during a 5 minute incubation period. In these conditions, purified Igs from two different C3 sera (E7 and F2) did not significantly affect ciliary beat frequency (Fig. 5A) while C2 purified Igs systematically reduced it when applied at 125 \( \mu g.mL^{-1} \) (Fig. 5A, a). In parallel, epithelial spheroids were incubated with C2 or C3 purified Igs after demembranation/ reactivation and were fixed after the antibody incubation for an immunofluorescence study (Fig. 5B). Both C2 and C3 Igs specifically located to centriole/basal bodies, demonstrating that both Igs have access to their epitopes in these conditions.

**DISCUSSION**

In this work, we have investigated the localisation and expression of centrin isoforms during in vitro mucociliary differentiation of human nasal epithelial cells using a primary culture method previously described (Jorissen et al., 1991). This cell culture provided an efficient model to characterise centriolar and/or cilia components at the biochemical and molecular level during the process of ciliated cell differentiation.

**Fig. 3.** Double immunofluorescence of epithelial spheroids during differentiation (image projections in laser confocal microscopy). Epithelial spheroids were fixed in PFA and processed for immunofluorescence using C2 (a and c), C3 (e) and GT335 (b,d,f). C2 decorates the centrioles of non-ciliated epithelial cells (a) and the centriole/basal bodies of ciliated cells (c). C3 also decorated the centriole/basal bodies of ciliated cells (e). C2 (c) and C3 (e) also stained cytoplasmic structures (arrowheads) which were only faintly recognised with GT335. a+b, c+d, e+f correspond to superimposition of both labellings. Bar, 10 \( \mu m \).

**Characterisation of centrin isoforms in human respiratory epithelial cells**

Centrin was first described as a component of basal bodies and contractile structures in unicellular green algae (Salisbury et al., 1984) and further identified so far in centriole/basal bodies of mammalian ciliated cells (Levy et al., 1996). Human centrin has been characterised using monoclonal and polyclonal antibodies raised against unicellular green algae centrin (Sanders and Salisbury, 1994; Paoletti et al., 1996; LeDizet et al., 1998). Nevertheless, these antibodies recognised the three human centrin gene products. In this work, we used specific polyclonal antibodies generated against recombinant HsCen2p and HsCen3p. C2 recognised two polypeptides with a molecular mass of about 20 kDa (see Fig. 1). We have been unable to distinguish between the two centrin isoforms 1 and 2, as a serum generated against recombinant HsCen1p detected the same polypeptide doublet (data not shown), although with different respective intensity for the two components. Thus, it is assumed that the slower migrating polypeptide corresponds to HsCen1p (Wiech et al., 1996). 2-D-electrophoresis revealed the presence of several isoforms detected with C2 serum, and the same pattern was observed in both native (data not shown) and in vitro differentiated epithelial cells (see Fig. 2), which corresponds to centrin 1 and centrin 2 isoforms and posttranslational modifications. The presence of two centrin isoforms in green algae which differed by their phosphorylation state has been previously suggested (Salisbury
The degree of centrin phosphorylation was shown to be clearly required either for the contraction or for the extension of the flagellar root and was also affected by Ca\(^{2+}\) (Salisbury et al., 1984, 1988; Martindale and Salisbury, 1990). In human nasal epithelial cells, Ca\(^{2+}\) influenced HsCen3p migration in SDS-polyacrylamide gels (see Fig. 1B), confirming its ability to alter centrin migration as shown for *Chlamydomonas* centrin and for Cdc31p (Salisbury et al., 1984; Spang et al., 1993). A mutant form of HsCen3p, where three amino acids of the fourth EF-hand have been replaced, inhibiting its Ca\(^{2+}\)-binding properties, shows different electrophoretic properties than the wild-type protein (Middendorp et al., 2000). By contrast, Ca\(^{2+}\) did not significantly modify the centrin pattern detected with C2 (see Fig. 1). These observations are in agreement with findings which documented differential conformational changes of centrin proteins upon Ca\(^{2+}\) binding (Wiech et al., 1996). Thus, protein pattern and biochemical properties clearly distinguish HsCen1/2p from HsCen3p.

**Differential human centrin gene transcription in human nasal epithelial cells**

Analyses by RT-PCR of the relative abundance of centrin mRNA with the use of specific primers clearly indicated that the 3 centrin mRNAs were expressed in human nasal epithelial cells. This is in contradiction with a recent study reporting that *HsCEN1* was never transcribed, both in fresh and in cultured human tracheal cells (LeDizet et al., 1998). This discrepancy could be explained by a difference in gene expression in tracheal and nasal epithelial cells. *HsCEN1* transcripts were indeed not detected during epithelial cell proliferation, but specifically expressed during differentiation while *HsCEN2* and *HsCEN3* mRNAs were detected during both phases (see Fig. 2). Additionally, non saturating PCR amplifications showed a significant and gradual increase in the expression of the 3 mRNAs. Thus, *HsCEN1* is apparently specifically expressed in ciliated cells while *HsCEN2* and *HsCEN3* are present in all epithelial cells. These observations are supported by previous reports carried out in rat, mouse and human tissues using specific primers for *HsCEN1* and *HsCEN2* (Wolfrum and Salisbury, 1998). *HsCEN2* was ubiquitously expressed while *HsCEN1* mRNA was specifically detected in testis and retina.

![Fig. 4](image_url) **Ultrastructural localisation of centrins in epithelial cells during ciliated cell differentiation.** Post-embedding immunogold localisation was performed using C2 (a-c) and C3 (a'-c'). C2 and C3 detect centrin proteins in electron-dense cytoplasmic granules (a and a', respectively) involved in the process of centriologenesis. Centrins are also localised in the elongating centrioles and migrating ones (arrows and arrowheads in b, b'). HsCen3p is clustered in the distal lumen of fully differentiated centriole/basal bodies (c') while HsCen1/2p extend in the proximal part of cilia axonemes (c). Bar, 0.3 μm.

Thus, it is likely that *HsCEN1* could correspond to an ‘axonemal’ isoform of centrin, specifically amplified during ciliated and flagellated cell differentiation.

**Centrin proteins are detected in the early process of ciliated cell differentiation**

In proliferative cells, the centriolar pathway involving the assembly of a new centrosome during each cell cycle suggests a direct structural continuity as new procentrioles appear to bud from the parental centrioles (Tournier and Bornens, 1994). The acentriolar pathway, which has been described during ciliated cell differentiation in vertebrate cells, involves the assembly of numerous centriole/basal bodies from undefined cytoplasmic structures which accumulate near the Golgi area (Dirksen, 1991). Biogenesis of centriole in both pathways is not understood at the molecular level but they are likely to involve common steps. A general conservation of some centriolar components among species has been largely documented. For example, proteins from yeast SPB such as Spc110p (Tassin et al., 1997), Spc98p (Tassin et al., 1998) and Cdc31p (Middendorp et al., 1997) show homologues in vertebrate cells. Among these components, centrin proteins are potentially important candidates for the common regulation of centriole assembly both in the centriolar and in the acentriolar pathway. In proliferative cells, centrin proteins could participate in the centrosome reproduction pathway or in cell cleavage as...
staining of midbodies has also been observed (Paoletti et al., 1996). In human epithelial cells, immunoelectron microscopy experiments show that centrin isoforms are abundant in fibrogranular aggregates and elongating centrioles (see Fig. 4), suggesting that centrin proteins could directly participate in centriole assembly (Fig. 6, for a scheme corresponding to ciliated cell differentiation and the localisation of HsCen1/2p and HsCen3p demonstrated in this work). These are the first centriolar proteins detected so far in the process of ciliated cell differentiation, suggesting an early role of these proteins in centriologenesis, before centriolar microtubule assembly. As a matter of fact, the centrin gene in Naegleria was the earliest one so far identified during differentiation (Levy et al., 1998). Interestingly, although colocalising in precursor structures, centrin isoforms eventually redistribute in distinct localisation in the fully assembled centriole/basal bodies.

**Two divergent centrin families displaying different cellular functions**

The existence of two divergent centrin subfamilies has been recently suggested (Middendorp et al., 1997). HsCen3p shows only 50% identity to CrCenp while HsCen1p and HsCen2p show 68-71% identity. Previous IF studies have shown that part of human centrin was associated with centrosomes or centriole/basal bodies in vertebrate cells (Levy et al., 1996; Paoletti et al., 1996; Middendorp et al., 1997; LeDizet et al., 1998; Wolfrum and Salisbury, 1998). More specifically, centrin proteins were located in the distal lumen of centrioles (Paoletti et al., 1996; LeDizet et al., 1998). In human epithelial cells, C2 and C3 decorated the centrosome of non-ciliated cells as well as centriole/basal bodies of ciliated cells. In mature ciliated cells, centrin proteins were localised in distal end of centriole/basal bodies but with a slightly distinct distribution: HsCen3p was strictly localised in the distal lumen of centriole/basal bodies while HsCen1/2p was also detected in the proximal part of the axonemes (Fig. 6).

In green algae, centrin has been associated with stellate fibres that link the doublet microtubules to the transition cylinder in the flagellar transition region (Schulze et al., 1987; Salisbury et al., 1988). These fibres are involved in the active severing of flagellar microtubules (Lewin and Lee, 1985; Sanders and Salisbury, 1989, 1994). Flagellar excision is mediated by a Ca$^{2+}$-induced contraction of centrin-based fibres of the transition zone of the axoneme. Monoclonal anti-centrin antibodies blocked the contraction of the transition zone, and Vfl-2, a *Chlamydomonas* mutant, which does not possess centrin-based fibre structures, was defective in normal excision mechanism (Sanders and Salisbury, 1994). Based on these results and on our ultrastructural localisation data, we have investigated a potential role for human centrins in cilia-associated functions. Intracellular Ca$^{2+}$ concentration [[Ca$^{2+}$]$_i$] is essential for ciliary beating, with a rise in [[Ca$^{2+}$]$_i$] correlating with a rise in ciliary beat frequency (Braiman et al., 1998, and references therein), and *Chlamydomonas* axonemes were shown to contain centrin-dynein-actin complexes (Piperno et al., 1992). Purified C2 Igs significantly decreased ciliary beat frequency and HsCen1p and/or HsCen2p are located in the proximal part of the axonemes. Consequently, centrin 1 and/or centrin 2 could be involved in the contractile function of cilia. By contrast, C3 Igs bound to their specific epitopes (see Fig. 5B) but did not modified ciliary beat frequency,
suggesting either that the antibody is not blocking or that HsCen3p is not involved in ciliary beating and should be rather involved in the centriole/basal body structure and/or assembly. This view is in agreement with another study showing that HsCen3p participates in centrosome duplication in animal cells (Middendorp et al., 2000). HsCen2p impaired cytokinesis in Xenopus embryos (Pauwels et al., 1996) while ectopic expression of HsCen3p resulted in undercleavage and inhibition of centrosome duplication. These results are consistent with findings in yeast which showed that Cdc31p, the most related protein to HsCen3p, is required for spindle pole body duplication (Baum et al., 1986).

In conclusion, differential expression and localisation of human centrin isoforms suggest a specific function for each protein. The specific expression of HsCEN1 during mucociliary differentiation and the inhibiting effect of C2 on ciliary beating suggest a requirement of HsCen1p and/or HsCen2p in centriole/basal bodies and cilia associated functions. Further molecular and biochemical studies using the cell system and antibodies described here will increase our understanding of the specific centrin functions. The most important result is the demonstration of centrin protein accumulation in fibrogranular precursors of centriole/basal bodies. This is the first protein identified in this precursor material. It provides a potential way to purify these structures using affinity approach. This should allow one to decipher the biochemical pathway for centriole assembly.

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