Primary cilia of human endothelial cells disassemble under laminar shear stress

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We identified primary cilia and centrosomes in cultured human umbilical vein endothelial cells (HUVEC) by antibodies to acetyl-α-tubulin and capillary morphogenesis gene-1 product (CMG-1), a human homologue of the intraflagellar transport (IFT) protein IFT-71 in Chlamydomonas. CMG-1 was present in particles along primary cilia of HUVEC at interphase and around the oldest basal body/centriole at interphase and mitosis. To study the response of primary cilia and centrosomes to mechanical stimuli, we exposed cultured HUVEC to laminar shear stress (LSS). Under LSS, all primary cilia disassembled, and centrosomes were deprived of CMG-1. We conclude that the exposure to LSS ends the IFT in cultured endothelial cells.

Introduction

We identified capillary morphogenesis gene-1 product (CMG-1) as the human homologue of IFT-71, a complex B protein supporting intraflagellar transport (IFT) in Chlamydomonas (Iomini et al., 2001). The antibodies against CMG-1 allowed us to detect primary cilia in cultured human umbilical vein endothelial cells (HUVEC) and find conditions eliciting the disassembly of these cilia.

Factors determining assembly and disassembly of primary cilia are unknown. Primary cilia respond to chemical and physical stimuli (Pazour and Witman, 2003) and regulate tissue morphogenesis in vertebrates (Nonaka et al., 1998; Nauli et al., 2003). They are composed of 3–30-μm-long, 9+0 axonemes stemming from the oldest centrioles of interphase cells from all tissues listed in http://members.global2000.net/bowser/cilialist.html. They disassemble at mitosis (Wheatley et al., 1996).

The IFT is required for assembly and maintenance of Chlamydomonas flagella and occurs in cilia and flagella regardless of their motility or differentiation (Rosenbaum and Witman, 2002). The IFT machinery is composed of kinesin II (Kozminski et al., 1995; Piperno et al., 1996), cytoplasmic dynein (Pazour et al., 1999a), and protein complexes (Pazour and Mead, 1997), referred to as IFT complex A and IFT complex B (Cole et al., 1998).

CMG-1 could be induced in HUVEC during vasculogenesis but was not located in any cellular compartment or identified as an IFT component. CMG-1 was previously cloned from a differential display cDNA library generated from HUVEC at different stages of capillary morphogenesis in vitro (Bell et al., 2001).

Primary cilia were not detected in endothelial cells in culture (Wheatley et al., 1996) but could be expressed in these cells for the following reasons. Primary cilia were observed in human aorta by electron microscopy (Bystrevskaya et al., 1992). Furthermore, polycystin-1 (PKD-1), a membrane protein mutated in polycystic kidney disease, is concentrated in primary cilia of various cells (Barr et al., 2001) (Yoder et al., 2002), including kidney cells. Finally, mutations in PKD-1 affect the formation of capillaries in pkd-1 mice (Kim et al., 2000).

Results and discussion

To identify a component of the IFT machinery in human, we initially analyzed the gene encoding Chlamydomonas IFT-71. We used amino acid sequences of seven peptides

Abbreviations used in this paper: CMG-1, capillary morphogenesis gene-1 product; HUVEC, human umbilical vein endothelial cells; IFT, intraflagellar transport; LSS, laminar shear stress.
(sequences surrounded by lines in Fig. 1 A) from the most abundant isoform of IFT-71 (see arrow in Fig. 2 A’) for the identification of a full-length cDNA clone. The IFT-71 cDNA encodes a protein with molecular weight 71,540 D and isoelectric point 8.98 in agreement with the migration of IFT-71 in two-dimensional PAGE (Piperno et al., 1998). The nucleotide sequence of IFT-71 is single copy, contains nine introns (Fig. 1 B), and likely expresses only one IFT-71 RNA, as assessed by Northern blot (not depicted). Therefore, the six isoforms of IFT-71 that were identified by a monoclonal antibody to IFT-71 (IFT-71ab) (Iomini et al., 2001; Fig. 2 A’) are likely products of posttranslational modification.

To confirm the identification of IFT-71 as an IFT protein in Chlamydomonas, we prepared a polyclonal antibody to his-tagged IFT-71 (hisIFT-71Ab) and used the antibody to analyze flagellar extracts and whole cells. The sedimentation profile of IFT-71 from a 35S-labeled flagellar extract, as revealed by Western blot (Fig. 2 B’), coincided with the sedimentation peak of IFT complex A and complex B (Fig. 2 B). Immunofluorescence by hisIFT-71Ab in cells exposed at the permissive temperature of 21°C was barely detectable along flagella of wild type and fla10-1, a thermosensitive mutant of anterograde IFT (Iomini et al., 2001; Fig. 2, C’ and D’, respectively). In contrast, hisIFT-71Ab was concentrated in bulges along flagella of fla15 and dhc1b, temperature-sensitive mutants of retrograde IFT (Piperno et al., 1998; Pazour et al., 1999b; Fig. 2, E’ and F’). Similar phenotypes of wild type, fla10-1, and fla15 were observed previously with antibodies to other subunits of the IFT machinery (Iomini et al., 2001).

IFT-71 is similar (NCBI, BLAST: 22% identical and 43% positive, E = 1e-39) to a Caenorhabditis elegans predicted protein C18H9.8, henceforth referred to as Ce-IFT-71. It is also similar (24% identical and 49% positive, E = 2e-36) to a human protein referred to as CMG-1 (Bell et al., 2001). Finally, CMG-1 is nearly identical (87.8% identical and 94.0% positive) to the mouse protein Mm-CMG-1, which we assembled from two predicted proteins, BAC35365.1 and NP_080595.1 (Fig. 1 A). These similarities extend throughout the full length of CMG-1, Ce-IFT-71, and Mm-CMG-1 proteins, and include several coiled-coil structures in similar positions, as predicted by SMART (not depicted).

Ce-IFT-71 is most likely a component of the IFT machinery. A regulatory element of IFT complex B genes in C. elegans (Swoboda et al., 2000) is present in a predicted intron of Ce-IFT-71 (Haycraft, C.J., personal communication). In contrast, the function and intracellular location of CMG-1 are unknown. Expression of a CMG-1–GFP chimera in HUVEC under control of an exogenous promoter failed to identify the intracellular location of CMG-1 (Bell et al., 2001).

To test the hypothesis that CMG-1 is a component of the IFT machinery, we tested whether CMG-1 is located in primary cilia. In turn, to identify primary cilia in HUVEC, we used a monoclonal antibody specific for acetyl-α-tubulin (AcTubab). Acetyl-α-tubulin is a major component of both primary cilia and centrioles (Piperno et al., 1987). Every HUVEC at interphase contained acetylated microtubules located mainly around the centrosomal region (Fig. 3 A). In addition, 5–35% of HUVEC expressed a primary cilium that was distinguished as a 4–5-μm rod containing the high-
PKD-1, a protein mediating mechanosensation in primary spond to mechanical stimuli, we identified the location of centrioles independently from the activity of IFT. Components at mitosis remain associated with the basal bodies/centrioles (Cole et al., 1998). This CMG-1Ab was detected around the centrosomal region of all HUVEC (Fig. 4, A and A', insets) by an antibody specific for the COOH terminus of the protein (Wilson et al., 1999). PKD-1 elicits a response to mechanical stimuli only in a subset of the HUVEC expressing PKD-1. We found PKD-1 concentrated in the centrosomal region of all HUVEC (Fig. 4, A and A') and around both centrioles (Fig. 4, B and B', insets) by an antibody specific for the COOH terminus of the protein (Wilson et al., 1999). PKD-1 was also located in primary cilia of <10% of ciliated HUVEC, where it was found in particles along the cilia that resembled those stained by hisCMG-1Ab (Fig. 4, A and A', insets; Fig. 4, B and B'). Therefore, PKD-1 elicits a response to mechanical stimuli only in a subset of the HUVEC expressing primary cilia if it functions as mechanosensor.

To test the effects of mechanical stress on primary cilia, confluent HUVEC monolayers were subjected to laminar shear stress (LSS) in a flow chamber receiving a constant flow of medium for various times. Subsequently, HUVEC were scored for number of cilia and intracellular location of CMG-1. To ensure physiological relevance, a level of LSS of 15 dyn/cm², similar to that generated in arterial circulation, was chosen.

In a typical experiment, primary cilia of HUVEC under LSS were maintained for 1 h but totally disassembled in the following hour. On the other hand, CMG-1 became undetectable in the majority of primary cilia within 1 h and in the centrosomal region in the following hour (Table I). After 30-min and 1-h intervals, we detected CMG-1 along one primary cilium but not along a second cilium in HUVEC. PKD-1 was concentrated around both spinelle poles at mitosis (Fig. 3, E', E", F', and F").

CMG-1, similar to IFT-71, was resolved in multiple isoforms in Western blots of proteins from HUVEC and mouse testes (unpublished data). Also it was part of 15–19S complexes from extracts of primary cilia of kidney cells (Nauli et al., 2003; Praetorius et al., 2003). We found PKD-1 concentrated in the centrosomal region of all HUVEC (Fig. 4, A and A') and around both centrioles (Fig. 4, A and A', insets) by an antibody specific for the COOH terminus of the protein (Wilson et al., 1999). PKD-1 was also located in primary cilia of <10% of ciliated HUVEC, where it was found in particles along the cilia that resembled those stained by hisCMG-1Ab (Fig. 4, A and A', insets; Fig. 4, B and B'). Therefore, PKD-1 elicits a response to mechanical stimuli only in a subset of the HUVEC expressing primary cilia if it functions as mechanosensor.
creased, and HUVEC oriented themselves in the direction of LSS with the centrosomal region often trailing from the leading edge of the cell (Fig. 5 C, C', and C '').

These observations suggest that HUVEC respond to LSS with the termination of IFT and the disassembly of primary cilia. They also suggest that ciliary PKD-1 in HUVEC either was undetected in the majority of primary cilia or is not required for the disassembly of primary cilia.

The disassembly of HUVEC primary cilia under LSS was similar in two aspects to the disassembly of flagella exposed at the restrictive temperature in \textit{fla}, temperature-sensitive mutants of flagellar assembly in \textit{Chlamydomonas}. First, components of the IFT machinery became undetectable along the axoneme within 1 h of exposure. Second, the axonemes disassembled in the following hour (Piperno and Mead, 1997; Iomini et al., 2001). On the other hand, components of the IFT machinery remained associated with the basal bodies/centrioles of the \textit{fla} at the restrictive temperature, whereas CMG-1, the putative IFT component of HUVEC primary cilia, became undetectable under LSS. Therefore, HUVEC oldest centrioles did not maintain or initiate the assembly of primary cilia under LSS.

The disassembly of primary cilia of HUVEC under LSS was part of a major rearrangement of the cytoskeleton. It coincided with changes in the acetylation of microtubules and in the organization of microfilaments (Wojciak-Stothard and Ridley, 2003). It was complete and not partial, as observed for primary cilia subjected to low temperature or trypsinization (Wheatley et al., 1996) or flagella under condition of chemical or mechanical stimulation (Lefebvre and Rosenbaum, 1986).

Membrane domains in direct contact with primary cilia or centrosomes may be sites for induction of specific signals that reorganize the cytoskeleton and cause a new orientation of HUVEC. If the expression of CMG-1 is increased during capillary morphogenesis in vitro (Bell et al., 2001), primary cilia may also have an important role in vasculogenesis.

\textbf{Materials and methods}

\textit{Chlamydomonas} strains and cell lines

\textit{Chlamydomonas} strains 137', \textit{fla10-1}, and \textit{fla15} were from our collection. \textit{dhc1b} was provided by G. Pazour and G. Witman (University of Massachusetts Medical School, Worcester, MA). HUVEC were from Cambrex Bio Science Walkersville, Inc. They were cultured in medium M199...
was generated using Superscript Choice System Kit (Invitrogen). Degenerate primers 5′-CAGGAGGCCTCTGCIAGTCAACACGCTGCTGAAAG-3′ and 5′-CTTSSAAAGACTCTGCCATCTCGAASACCCGCCS5W-3′, where \( Y = C + T, R = A + G, W = A + T, S = G + C, \) and \( I = \text{inosine} \), were designed from peptides QELADYNTVLDK and SAGVFEMDEFLK, respectively, which were identified by mass spectrometry.

The cDNA fragment was used to screen a deflagellation cDNA library (Chlamydomonas genetic center, project ID 1030). Out of the nine positives clones identified by Southern blot, two were sequenced. The open reading frames in these clones encoded a protein of 641 amino acids (Fig. 1 A). The GenBank/EMBL/DBJ submission number of the cDNA sequence of IFT-71 is AY505143. The open reading frames of IFT-71 cDNA exactly matched the sequences corresponding to 10 predicted exons of IFT-71, as reported in the database of the Chlamydomonas genome available at ftp://ftp.jgi-psf.org/pub/JGI_data/Chlamy, see scaffold 246.

Following the identification of IFT-71, a BLAST search of the GenBank/DMBL/DBJ database showed that IFT-71 cDNA is almost identical to the entry AY245434 [intraflagellar transport protein component IFT74/72] submitted by others (Qin et al., 2004). However, the sequence reported in AY245434 diverged by 10 nucleotides in the coding region from the predicted exons of IFT-71. As a consequence, the translated protein differed by 10 amino acids from the sequence that we predicted.

For Northern blot analysis, 3 μg of poly(A)−RNA was run on an agarose gel and transferred to Gene screen Plus hybridization membrane (NEN Life Science Products) following standard methods. Probes were labeled with [32P] using the random Prime-It Kit (Stratagene). The cDNA coding regions of both IFT-71 and CMG-1 were cloned into a pQE-30 bacterial expression vector using the KpnI and HindIII restriction sites at the 5′ ends, which were identified by mass spectrometry.

**LSS**

LSS was applied to HUVEC for times ranging from 30 min to 15 h using a parallel plate flow chamber set in a incubator with 5% CO₂ at 37°C. A peristaltic pump and a depulsator (Nonaka et al., 2002) delivered a constant flow of medium to the chamber to obtain 15 dyn/cm². The cross section of the flow chamber was 0.5 × 0.034 cm. Wall shear stress \( \tau \) was calculated using the equation \( 3\rho Q2ab \) (Houston et al., 1999), where \( y \) is viscosity (poise) of the medium at 37°C, \( Q \) is the volumetric flow rate (ml/s), \( a \) is the half channel height (cm), and \( b \) is the channel width (cm).

**Preparation and use of antibodies**

Preparation and specificity of the monoclonal antibody IFT-71ab were previously described (Iomini et al., 2001). Binding of this antibody provided a signal in Western blot but not in immunofluorescence of Chlamydomonas cells.

The two bacterial proteins hisIFT-71 and hisCMG-1 were affinity purified on an Ni-NTA agarose bead column (QIAGEN) and injected in rabbits by Covance Research Products Inc. The cDNA coding regions of both IFT-71 and CMG-1 were cloned into a pQE-30 bacterial expression vector using the KpnI and HindIII restriction sites at the 5′ and 3′ ends, respectively, created by PCR.

**Other procedures**

Microscopy of Chlamydomonas and HUVEC was performed with a Leica TCS-SP (UV) confocal laser scanning microscope in an inverted configuration as previously described (Iomini et al., 2001). Images were processed by Adobe Photoshop 5.5®.

We isolated IFT complex A and complex B from the remaining proteins by the same procedure as previously described (Iomini et al., 2001). Images were processed by Adobe Photoshop 5.5®. We identified IFT complex A and complex B from the remaining proteins of the flagellar matrix and resolved all subunits of the IFT complexes as previously described (Piperno et al., 1998). Chlamydomonas culture, 35S labeling of proteins, gel electrophoresis, and Western blots were performed as previously described (Piperno et al., 1998).

Mouse testes were isolated, washed in PBS, frozen, and thin sliced. Extraction of the slices was performed with 0.3 ml/testes of 0.05 M NaCl, 4

**Table 1. Primary cilia of HUVEC disassemble under LSS**

| Exposure time to LSS | Primary cilia identified by ActTubab | Primary cilia identified by hisCMG-1Ab | Centrosomes identified by hisCMG-1Ab |
|---------------------|-------------------------------------|----------------------------------------|-------------------------------------|
| 0                   | 8.0                                 | 7.6                                    | 100                                 |
| 0.5 h               | 9.0                                 | 7.0                                    | 100                                 |
| 1 h                 | 7.3                                 | 2.6                                    | 100                                 |
| 2 h                 | 0                                   | 0                                      | 0                                   |

300 HUVEC were analyzed. Numbers are percentages.
mM MgCl₂, 0.001 M Hepes, pH 7.2, containing proteolysis inhibitors “Complete Mini” (Roche Diagnostics) one tablet/10 ml. The suspension was exposed to ultrasound, spun at 14,000 rpm for 10 min in a minifuge Eppendorf 5415 C at 4°C. 200 μl of the supernatant was resolved as Chlamydomonas flagellar extracts (Piperno et al., 1998). This report is in memory of our colleague Massimo Sassaroli who died on July 6, 2003.

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