[Frontiers in Bioscience 12, 1661-1669, January 1, 2007]

Identification of ICIS-1, a new protein involved in cilia stability

Cecile Ponsard [1], Marie Skowron-Zwarg [1], Virginie Seltzer[1], Eric Perret [2], Julia Gallinger [3], Cathy Fisch [3], Pascale Dupuis-Williams [3, 4], Nathalie Caruso [1], Sandrine Middendorp [1], and Frederic Tournier [1]

[1] Laboratoire de Cytophysiologie et Toxicologie Cellulaire, Universite Paris 7, France, [2] Sanofi-Aventis, Labege, France, [3.] UMR 8080, Centre National de la Recherche Scientifique/Universite Paris Sud, Orsay, France, [4] Ecole Superieure de Physique et Chimie Industrielles, Paris, France

TABLE OF CONTENTS

1. ABSTRACT

Cilia are specialized organelles that exert critical functions in numerous organisms, including that of cell motility, fluid transport and protozoan locomotion. Ciliary architecture and function strictly depend on basal body formation, migration and axoneme elongation. Numerous ultrastructural studies have been undertaken in different species to elucidate the process of ciliogenesis. Recent analyses have led to identification of genes specifically expressed in ciliated organisms, but most proteins involved in ciliogenesis remain uncharacterized. Using human nasal epithelial cells capable of ciliary differentiation *in vitro*, differential display was carried out to identify new proteins associated with ciliogenesis. We isolated a new gene, ICIS-1 (Involved in CIlia Stability-1), upregulated during mucociliary differentiation. This gene is localized within the TGF-beta1 promoter and is ubiquitously expressed in human tissues. Functional analyses of gene expression inhibition by RNA interference in *Paramecium tetraurelia* indicated that the ICIS-1 homologue interfered with cilia stability or formation. These findings demonstrate that ICIS-1 is a new protein associated with ciliated cells and potentially related to cilia stability.

2. INTRODUCTION

Cilia or flagella play clear-cut roles in numerous organisms, including that of cell motility of sperm, transport of mucus along the respiratory tract and stirring of the cerebrospinal fluid in vertebrates, and locomotion and nutrition in protists. Numerous ultrastructural studies have been undertaken in different species to describe the particular process of ciliogenesis (1). Two pathways of basal body assembly exist. In epithelial cells, procentrioles are assembled *de novo* around electron-dense cytoplasmic granules near the Golgi area. Centrioles elongate until they are full-length, then migrate via an actin-dependant mechanism and anchor to the apical membrane where they trigger axonemal microtubule polymerization and cilia formation. In contrast, in ciliated protozoan, basal bodies duplicate: procentrioles elongate perpendicularly to their parents and then anchor to the cortex before cilia assembly. Nevertheless, most of the 250 proteins localized within centrioles or basal bodies remain uncharacterized (2). Recent genomic analyses led to the identification of genes specifically expressed in ciliated organisms (3, 4), and recent proteomic studies enabled identification of the 150 proteins specifically localized within centrioles (3-6).

Comparison of different cellular models is also useful for identifying new proteins. Among these models, primary cultures of human respiratory epithelial cells permit characterization of proteins expressed during ciliated cell differentiation (7).

Alterations in human airway pseudostratified epithelium can be observed in several genetic disorders, including primary cilia dyskinesis (PCD) characterized by a defect in cilia formation or function (8), and in several inflammatory diseases, including asthma and cystic fibrosis (9). The inflammatory cytokine interleukin-13 (IL-13) is a key regulator of Th2-dominated disorders such as asthma (10). IL-13 overexpression in transgenic mice causes subepithelial airway fibrosis similar to the fibrotic response observed in human asthmatic airways (11). Furthermore, this inflammatory cytokine influences mucociliary differentiation (MCD) of human nasal epithelial (HNE) cells *in vitro* towards a secretory pathway. IL-13 increases the proportion of secretory cells and inhibits ciliated cell differentiation (12) (a condition hereafter referred to as McD) compared to MCD observed in the absence of IL-13, leading to a higher proportion of ciliated cells (a condition hereafter referred to as mCD). Finally, it was recently shown that IL-13 could stimulate differentiation of ciliated cells into goblet cells, involving EGFR activation (13).

To identify new genes up- or downregulated during MCD, we used primary cultures of HNE cells and performed cDNA differential display using cell spheroids maintained for 9 days of differentiation in the presence or absence of IL-13 (12). We showed that TGF-beta1 was upregulated during McD, while a new gene located within the TGF-beta1 promoter was downregulated. This new gene, ICIS-1 (Involved in Cilia Stability-1) is widely conserved during evolution. ICIS-1 is gradually expressed during mCD (-IL-13) and its expression is inhibited during McD (+IL-13). Moreover, RNAi experiments in *Paramecium tetraurelia* led to cilia disruption, suggesting that the ICIS-1 homologue is involved in cilia stability.

3. MATERIALS AND METHODS

3.1. Cell culture

Primary cell culture was achieved as initially described in Laoukili *et al*., 2001 (12). Briefly, human nasal polyps or turbinates were surgically removed, and epithelial cells were dissociated from the tissue by pronase. After pre-plating, the cell suspension was dispersed on type I collagen gels in T-25 culture flasks. The medium was replaced three times a week. After 2 weeks (cell confluence), collagenase was added to resolve the collagen gel and to release the epithelial cells as cell sheets in suspension, pipetted into T-25 uncoated culture flasks. Cells were placed on a continuous rotating shaker (80 rpm) at 37°C and cell sheets formed stable aggregates (epithelial spheroids). During the latter step, epithelial cells polarized and some differentiated into secretory and ciliated cells. The proportion of differentiated cell types was estimated by western blot or flow cytometry using specific antibodies (see below and (12)).

3.2. Cytokine and antibodies

Human recombinant IL-13 was purchased from R&D Systems Europe Ltd. (Abingdon, United Kingdom). GT335, a monoclonal antibody raised against a chemically glutamylated C-terminal peptide of alpha-tubulin (14), was a gift from P. Denoulet (Université Paris 6, Paris, France). To obtain antibodies directed against ICIS-1, six histidine-tagged protein was purified on nickel-chelated agarose beads and injected into two rabbits according to Agrobio (La Ferté Saint Aubin, France) protocol.

3.3. cDNA differential display

Total RNA was extracted from a primary culture of epithelial cells treated or non-treated with IL-13 at day 11 during differentiation, according to Chomczynsky and Sacchi, 1987 (15). Poly (A+) RNA was isolated using an oligo-dT kit (Qiagen) and cDNA was synthesized from 2 µg of mRNA. The cDNA differential display was performed using DNA subtraction kit PCR Select from Clontech, according to the manufacturer's instructions.

3.4. RT-PCR

Total RNA was extracted from cultures using the TriReagent® RNA isolation reagent (Sigma). Total RNA of 1 µg was reverse-transcribed. PCR amplifications were performed on 2 µl of cDNA with specific primers for GAPDH, TGF-beta1 and ICIS-1. Sequences used for each primer are available on request. PCR products were analyzed by electrophoresis on 2% agarose gel. Band intensity was compared with respect to the GAPDH mRNA content.

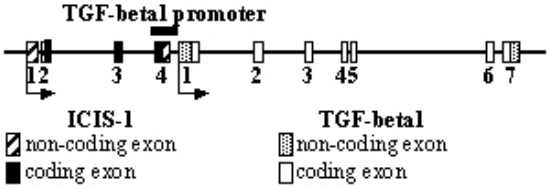
3.5. Blot analyses

Northern blot of human tissue mRNA was purchased from Clontech and hybridized with an ICIS-1-specific probe obtained by PCR and labeled with the redyprime II DNA labeling system (Amersham Biosciences) according to the manufacturer's instructions. The blot was pre-hybridized and hybridized in Rapid-Hyb solution (Amersham Biosciences) and washed according to the manufacturer's instructions.

For western blots, total protein extracts were prepared from HNE cells in SDS-PAGE sample buffer and resolved by electrophoresis in 10% SDS-PAGE. Immunodetection was performed as previously described (16) using GT335 mAb (1:10000) or an affinity-purified rabbit polyclonal antibody directed against human ICIS-1 (1:250). Secondary Ab coupled to peroxidase (Dako) and chemiluminescence development (Perkin Elmer) were used.

3.6. Gene silencing in *Paramecium tetraurelia*

Wild-type cells of stock d4-2 are a derivative of the wild-type stock 51 of *P. tetraurelia* (17). Cells were grown at 27°C in a buffered infusion of wheat grass powder (Pines international Co.), supplemented with 0.4 µg/ml b-sitosterol and inoculated with *Klebsiella pneumoniae*. The ICIS-1 sequence was cloned between two T7 promoters in the L4440 feeding vector (18), here modified with the Gateway®Vector Conversion System



A

```
ACCAAGACGAGCGCGGTCGCGGAAGTATCGCGGCTTACAT
ATCTCGCAGCCAGGCGGGTCCTGGGAGAGGCGCGAGCCAG
GCCTGGTCGGCGCCCGCGGAGAAAAGAAGCAGTCAAGCCC
ATGAACTACAACCCCGGTTGCCGCTTTCTCCTCTCCTAAC
CGTTAAGTGCGCTAAGGGCC ATG GCT GAG GTG CAC
                      M   A   E   V   H
 GTG ATC GGG CAG ATC ATA GGG GCC AGC GGT
  V   I   G   Q   I   I   G   A   S   G
TTC TCG GAA AGT AGC CTC TTC TGC AAG TGG
  F   S   E   S   S   L   F   C   K   W
GGC ATT CAC ACA GGG GCG GCA TGG AAG CTC
  G   I   H   T   G   A   A   W   K   L
CTG TCA GGC GTG CGG GAG GGC CAA ACG CAA
  L   S   G   V   R   E   G   Q   T   Q
GTG GAC ACC CCG CAG ATA GGG GAC ATG GCT
  V   D   T   P   Q   I   G   D   M   A
TAC TGG TCC CAC CCC ATC GAC CTG CAC TTC
  Y   W   S   H   P   I   D   L   H   F
GCC ACC AAA GGT CTT CAA GGC TGG CCC CGG
  A   T   K   G   L   Q   G   W   P   R
CTC CAT TTC CAG GTG TGG TCC CAG GAC AGC
  L   H   F   Q   V   W   S   Q   D   S
TTT GGC CGC TGC CAG CTT GCA GGC TAT GGA
  F   G   R   C   Q   L   A   G   Y   G
TTT TGC CAT GTG CCC AGT AGC CCG GGC ACC
  F   C   H   V   P   S   S   P   G   T
CAC CAG CTG GCC TGC CCC ACG TGG CGG CCC
  H   Q   L   A   C   P   T   W   R   P
CTG GGC AGT TGG CGA GAA CAG TTG GCA CGG
  L   G   S   W   R   E   Q   L   A   R
GCT TTC GTG GGT GGT GGG CCG CAG CTG CTG
  A   F   V   G   G   G   P   Q   L   L
CAT GGG GAC ACC ATC TAC AGT GGG GCC GAC
  H   G   D   T   I   Y   S   G   A   D
CGC TAT CGC CTG CAC ACA GCT GCT GGT GGC
  R   Y   R   L   H   T   A   A   G   G
ACC GTG CAC CTG GAG ATC GGC CTG CTG CTC
  T   V   H   L   E   I   G   L   L   L
CGC AAC TTC GAC CGC TAC GGC GTG GAG TGC
  R   N   F   D   R   Y   G   V   E   C
TGA GGGACTCTGCCTCCAACGTCACCACCATCCACACC
  *
CCGGACACCCATGATGGGGGAGGATGGCACAGTGGTCAAGA
GCACAGACTCTAGAGACTGTCAGAGCTGACCCCAGCTAAGG
CATGGCACCGCTTCTGTCCTTTCTAGGACCTCGGGGTCCCT
CTGGGCCCAGTTTCCCTATCTGTAAATTGGGGACAGTAAAT
GTATGGGGTCGCAGGGTGTTGAGTGACAGGAGGCTGCTTAG
CCACATGGGAGGTGCTCAGTAAAGGAGAGCAATTCTTGCAN
AAAAAAA
```

B

Figure 1. Sequence and organization of the ICIS-1 gene. A. Genomic organization of ICIS-1 and TGF-beta1 genes. TGF-beta1 promoter contains the fourth ICIS-1 exon. B. Nucleotide and deduced amino acid sequences of full-length ICIS-1 cDNA, revealed by 5' and 3' RACE. C2 and B9 domains are underlined by full line and dotted line, respectively.

(Invitrogen). The resulting construct was used for transformation of an Rnase-III-deficient strain of *Escherichia coli* with an IPTG-inducible T7 polymerase, HT115 (DE3) (19). RNAi experiments were carried out using the feeding method as previously described (20). In brief, individual cells were cultured in double-stranded RNA-expressing bacteria. Phenotypes were screened after 24 to 96 h. Control experiments were done by feeding the paramecia with HT115 bacteria carrying no plasmid.

Immunofluorescence experiments were performed as described by Dupuis-Williams *et al*., 2002 (20). We used a 1/10 dilution of culture supernatant of a monoclonal antibody, Axo 49 recognizing polyglycylated sites in alpha- and beta-tubulin localized in stable microtubules such as paramecium axonemes (21). Cells were analyzed with an epifluorescence microscope (Axiophot, Zeiss*)* coupled to an « Insight QE » (Spot) CCD camera..

4. RESULTS

4.1. ICIS-1 is a new gene localized within the TGF-beta1 promoter region

To identify new genes up- or downregulated during MCD, a cDNA differential display was performed to compare transcriptomes of HNE cells in the absence or presence of inflammatory cytokine IL-13 between day 2 and day 11. TGF-beta1 was upregulated during McD (+IL-13). This overexpression was also observed using an other cDNA differential display performed on differentiated HNE cells treated for 48 h with the inflammatory cytokine (data not shown). In parallel, several cDNA downregulated during McD were identified. Interestingly, one of them corresponded to an open reading frame (ORF) partially localized within the TGF-beta1 promoter (Figure 1A). To characterize this ORF, rapid amplifications of cDNA ends were performed using 5' and 3' RACE-PCR with specific primers and total cDNA samples from HNE cells. After determining mRNA 5' and 3' ends, the full-length cDNA was amplified by PCR (Figure 1B). The predicted amino acid sequence contains a C2 domain (4) involved in binding phospholipids in a calcium-dependent manner but which can also function in a calcium-independent manner mediating protein-protein interactions (22). The ICIS-1 protein sequence also shows significant similarity to the eukaryotic EPPB9 protein, identified in endothelial precursor cells, whose function remains unknown, but which was recently described as interacting with Rrp6 exonuclease, a protein involved in mRNA degradation (23).

Blast search analyses showed that the ICIS-1 gene is homologous to genomic sequences in species containing cilia or flagella, but absent from non-ciliated species such as *A. thaliana* (Figure 2). Interestingly, rat and mouse homologues are also located within the TGF-beta1 promoter (data not shown). In addition, this gene is homologous to two *Drosophila melanogaster* genes (NP_608998 and NP_650470), which were identified in genomic screening for selecting genes conserved in cilia-possessing species (4).Two homologue genes were also found in ciliated protozoans such as *P. tetraurelia*.

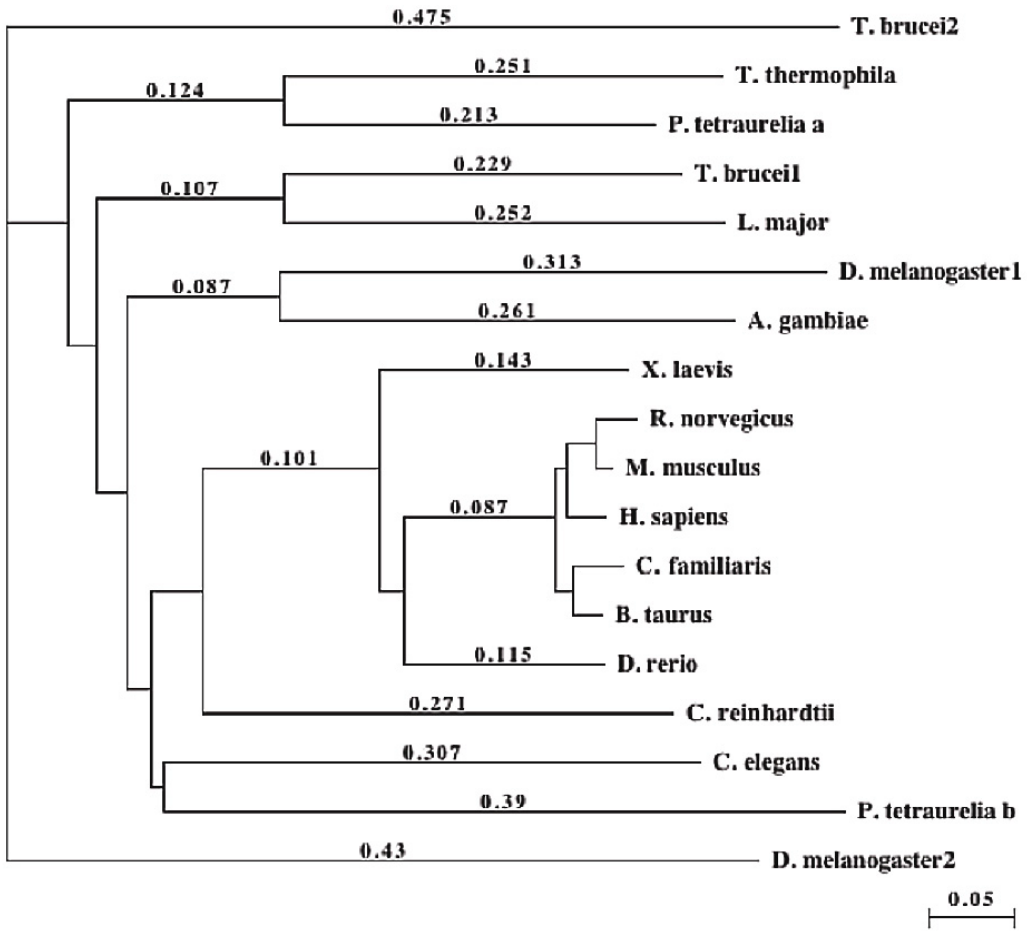


Figure 2. Phylogenetic tree of ICIS-1 homologues. Unrooted tree, showing phylogenetic distances, was obtained using ClustalW with the Blosum similarity matrix. Sequence accession numbers: *Anophela gambia* EAA11961, *Bos taurus* AAW82106, *Canis familiaris* XP_533658, *Caenorhabditis elegans* NP_500186, *Chlamydomonas reinhardtii* www.jgi.doe.gov/chlamy 137074, *Danio rerio* AAH76193, *Drosophila melanogaster* 1 NP_608998, *Drosophila melanogaster* 2 NP_650470, *Homo sapiens* AAH04444, *Leishmania major* CAJ04592, *Mus musculus* NP_742160, *Paramecium tetraurelia* a GSPATP00034796001, *Paramecium tetraurelia* b GSPATP00019571001, *Rattus norvegicus* XP_218345, *Tetrahymena thermophila* EAR89828, *Trypanosoma brucei* 1 XP_828130, *Trypanosoma brucei* 2 XP_825763, *Xenopus laevis* AAH73648.

4.2. ICIS-1 is ubiquitously expressed in human tissues and differentially expressed during MCD

To assess the tissue distribution of ICIS-1, northern blot analysis was carried out on total RNA corresponding to different human tissues using a specific ^{32}P-labeled ICIS-1 cDNA probe. ICIS-1 was expressed in lung, brain (ciliated cell-containing tissues) and pancreas, placenta, and heart (non-ciliated cell-containing tissues) with different levels of expression. A major form corresponding to 1 kb transcripts was detected (Figure 3, asterisk). A minor form of 2.5 kb was also observed, corresponding to a putative alternatively spliced isoform (Figure 3, arrowhead).

cDNA differential display identified the ICIS-1 gene as being upregulated during mCD, while TGF-beta1 was upregulated during McD. Furthermore, RT-PCR experiments were performed using RNA extracted from epithelial spheroids (18 days after confluence) treated for 24 h with increasing concentrations of the inflammatory cytokine. The ICIS-1 mRNA level decreased in a dose-dependent manner, while the TGF-beta1 mRNA level increased (Figure 4A). GAPDH was used as a housekeeping gene; its expression remained constant within cells (16) (Figure 4A, 4B). To test ICIS-1 gene expression during ciliogenesis, RT-PCR was performed on total RNA extracted from epithelial spheroids during MCD (Figure 4B). ICIS-1 gene expression was induced during differentiation (day 7 and day 12 compared to day 0) and slightly decreased at day 17 after confluence during mCD (-IL-13). In contrast, during McD, IL-13 decreased ICIS-1 gene expression.

To detect ICIS-1 protein expression during MCD, western blots were performed using affinity-purified ICIS-1 antibody (Figure 4C). ICIS-1 expression gradually increased during mCD (compare day 7 to day 17). Moreover, during McD, ICIS-1 expression detected by

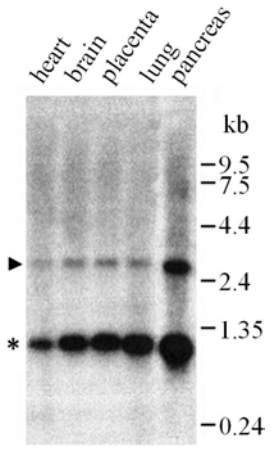


Figure 3. Expression of ICIS-1 in different human tissues. Northern blotting of different human tissues using the ICIS-1 cDNA probe reveals that ICIS-1 is expressed in heart, brain, placenta, lung and pancreas. Two ICIS-1 forms are detected, a major form corresponding to a 1 kb transcript (asterisk) and a minor form corresponding to a 2,5 kb transcript (arrowhead).

western blotting was largely reduced; in parallel, the proportion of ciliated cells detected by GT335 staining decreased. In another model of MCD, the air-liquid interface culture model (24), ICIS-1 protein expression was also enhanced parallel with ciliogenesis, and was not detected in non-ciliated cells (data not shown). Pre-adsorption of the purified antibody with the ICIS-1 recombinant protein abolished the signal, confirming the specificity of the polyclonal antibody. Our results indicated that ICIS-1 expression is correlated with the proportion of ciliated cells.

4.3. Gene expression inhibition of the ICIS-1 homologue in Paramecium by RNAi interferes with ciliogenesis

The function of ICIS-1 was studied by gene silencing in *P. tetraurelia,* which like all ciliated species, possesses ICIS-1 homologues (Figure 2). Homology-based searches in the paramecium genome resulted in identification of three ICIS-1 homologues, two of which encoded the same protein. The two ICIS-1 paramecium proteins, respectively, shared 36% (ICIS-1a) and 34% (ICIS-1b) identity with the human homologue (Figure 5A). Preliminary RNAi experiments with two individually silenced genes led to similar phenotypes, suggesting that both proteins are involved in the same physiological pathway. We thus focused our functional analysis on the *Pt*ICIS-1a protein, which shows a protein sequence closer to the human homologue (Figure 5A). RNAi experiments were performed by feeding paramecia with bacteria producing double-stranded RNA from the ICIS-1a gene cloned between two inducible promoters in a plasmid. Throughout the experiment, paramecia were subcloned daily and re-fed with freshly induced bacteria to maintain the RNAi effect (these cells are hereafter referred to as ICIS-1a$^-$). These cells were compared to control cells grown under the same conditions with bacteria lacking the plasmid (referred to as WT). Under these conditions, wild-type cells divided 3 to 4 times a day. At each cell division, basal bodies duplicated; the newly formed basal bodies rapidly acquired full-length cilia enabling the formation of two daughter cells, with complete ciliature before the completion of cytokinesis. The effect of RNAi was first studied by examining cell growth and behavior. The cell division cycle was significantly reduced in ICIS-1a$^-$ cells 24 h after the onset of feeding (Figure 5B). Moreover, after two days, lethality was observed to increase over the next few days (Figure 5C), probably accounting for the progressive reduction in the number of divisions. This result suggested that depletion of the ICIS-1a protein increased the generation time, while lethality was probably secondarily induced.

Observation of cells under stereo microscope revealed that, at 24 h, a high proportion of ICIS-1a$^-$ cells presented altered swimming while, after 48 h nearly 95% of cells exhibited this phenotype (data not shown). This can be interpreted as being the effect of depletion of the ICIS-1a protein, since 24 h is usually the time necessary for RNAi to take place and the intracellular pool of proteins to be depleted (20, 25, 26). Since this phenotype was suggestive of a problem of cilia organization or beating, cilia distribution and appearance were studied by indirect immunofluorescence using Axo 49, a mAb detecting polyglycylated tubulins contained in cilia (Figure 5D). Paramecia possess some 4000 cilia that are distributed ¾ in the cortex and ¼ in the oral apparatus, and whose coordinated beating ensures cell locomotion and feeding by moving the bacteria towards the oral cavity. In contrast to WT cells, ICIS-1a$^-$ cells displayed altered cilia distribution. The phenotype, although heterogeneous in severity, was characterized by a loss of cilia both in the cortex and in the oral apparatus, and by the presence of very short cilia. However, Axo 49 labeling of basal bodies revealed that basal body organization on the cortex was not disturbed, suggesting that the duplication process and anchoring to the cortex were not affected by ICIS-1a gene silencing. These experiments demonstrated that the ICIS-1 gene is directly involved in ciliogenesis or cilia stability.

5. DISCUSSION

Cilia absence or abnormalities have been reported in several diseases (8). Recent studies using genomic and proteomic approaches have been used to identify new genes encoding cilia proteins. In this paper, we used a cDNA differential display to identify genes expressed during MCD of HNE cells and regulated by a key inflammatory cytokine, which was shown to interfere with ciliated cell differentiation (12). We identified a new gene, ICIS-1, located within the TGF-beta1 promoter, conserved in cilia-containing species, upregulated during MCD and described as being necessary for cilia function and/or stability in paramecia.

5.1. Genomic localization of ICIS-1

ICIS-1 gene overlaps the TGF-beta1 promoter in humans and this unusual localization was also found in rat and mouse genomes. Overlapping genes in mammalian genomes are unexpected phenomena even though hundreds

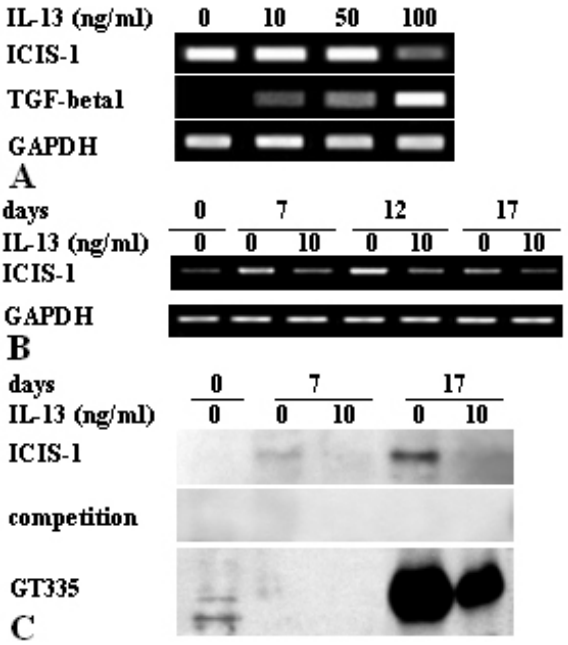


Figure 4. Expression of ICIS-1 and TGF-beta1 during MCD. A. ICIS-1 and TGF-beta1 gene expression in the presence of increased IL-13 concentrations. Epithelial spheroids at 18 days after confluence were treated for 24 h with increasing IL-13 concentrations. ICIS-1 mRNA level, shown by RT-PCR on total mRNA extracted to epithelial spheroids, is decreased in the presence of IL-13 in a dose-dependent manner whereas TGF-beta1 gene expression is stimulated. GAPDH, used as housekeeping gene, is stably expressed during MCD. B. Semi-quantitative RT-PCR analysis of ICIS-1 and TGF-beta1 gene expression (7, 12 and 17 days after confluence) in the absence or presence of IL-13. ICIS-1 gene expression is induced at day 7 and day 12, and decreases at day 17. When IL-13 is added, ICIS-1 gene expression is lower. GAPDH, used as constant marker, is not affected by IL-13 treatment. C. ICIS-1 protein expression during MCD in the presence or absence of IL-13. Immunoblotting of 10 µg of total protein extracted to epithelial spheroids with antibodies directed against human ICIS-1 protein shows that ICIS-1 expression is induced during MCD (in the absence of IL-13). Treatment with 10 ng/ml of IL-13 reduces ICIS-1 expression, which is not detected under our conditions. Competition tests (pre-adsorption of the purified antibody with the recombinant protein) abolish ICIS-1 signals, confirming the specificity of our antibodies. GT335, which is a marker of ciliated cells, shows that IL-13 alters the MCD decreasing GT335 signal.

of pairs of protein-coding overlapping genes have been reported thus far (27). Moreover, overlapping genes generally possess antisense direction of transcription; their involvement in physiological processes and gene regulation in living organisms is not fully understood. Interestingly, ICIS-1 and TGF-beta1 possess sense direction suggesting that ICIS-1 and TGF-beta1 genes could directly regulate each other. ICIS-1 transcription could inhibit fixation of specific transcription factors within the TGF-beta1 promoter region during ciliated cell differentiation. Conversely, fixation of TGF-beta1 transcription regulators could inhibit ICIS-1 transcription in the presence of the inflammatory cytokine IL-13. This hypothesis was reinforced by the differential expression of these two genes during MCD. Nevertheless, additional experiments are necessary to understand the transcriptional regulation of these two overlapping genes in mammalian cells.

5.2. Differing regulation of ICIS-1 and TGF-beta1 expression during MCD

In our experiments, we used the inflammatory cytokine IL-13, which influences MCD by reducing the proportion of ciliated cells and increasing the proportion of secretory cells (12). Furthermore, this cytokine is able to act directly on epithelial cells, and appears to be responsible for remodeling and development of fibrosis in inflamed airways (28).

Using a cDNA differential display, we showed that TGF-beta1 expression was enhanced during McD. TGF-beta1 is a pluripotent growth factor implicated in wound repair, proliferation and differentiation processes (29). Moreover, it has a potent profibrotic effect in different cell types, regulating expression of numerous matricial proteins (30, 31). Therefore, TGF-beta1 could be activated in the presence of IL-13 promoting epithelial fibrosis, as suggested in mice (31). Interestingly, unusually high levels of TGF-beta1 and IL-13 are detected in airways of asthmatic patients (32, 33). A previous study had shown that both TGF-beta1 and IL-13 may act in combination, for example to induce eotaxin-1 production (34), thereby reinforcing airway inflammation. Altogether, these results suggest that TGF-beta1 release, in response to IL-13 during inflammation, may be implicated in airway remodeling and that the two factors may cooperate to enhance an inflammatory response associated with respiratory diseases. In contrast, ICIS-1 mRNA was mainly expressed during mCD and the protein, detected using polyclonal antibodies directed against recombinant ICIS-1, gradually increased. Nevertheless, mRNA levels of ICIS-1 decreased at day 17, whereas ICIS-1 protein accumulated. This implies extensive mRNA production and protein accumulation when MCD starts. After ciliogenesis, ICIS-1 mRNA may be degraded, while the ICIS-1 protein, which is important for cilia function or stability (see below), remains present.

Thus, our results revealed an opposite and dose-dependent regulation of ICIS-1 and TGF-beta1, which might reflect their particular genomic localization. And, in opposition to TGF-beta1, ICIS-1 may not be involved in inflammatory response but preferentially behaves during ciliogenesis.

5.3. Is ICIS-1 involved in ciliogenesis?

Western blot experiments showed that ICIS-1 was not detected at cell confluence but gradually accumulated during mCD. Furthermore, expression of the ICIS-1 protein was downregulated upon IL-13 treatment which, as expected, also reduced the proportion of ciliated

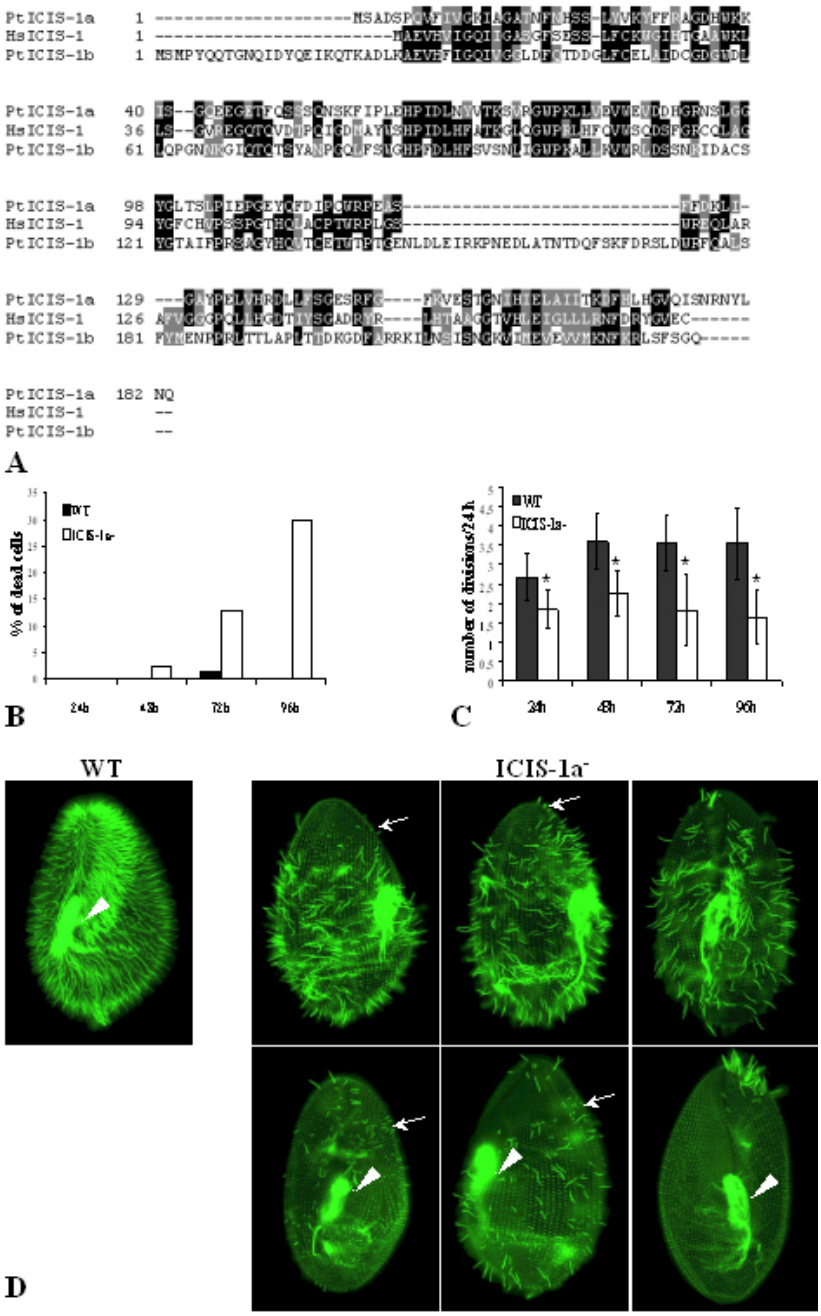


Figure 5. Inhibition of ICIS-1 homologue by RNAi in *P. tetraurelia*. A. Sequence comparison (boxshade program) of the human ICIS-1 protein (HsICIS-1) with the two paramecium proteins: PtICIS-1a (GSPATP00034796001) and PtICIS-1b (GSPATP00019571001). Identical amino acids present in at least two sequences are boxed in black and conservative changes are boxed in grey. B. Percentage of WT and ICIS-1a$^-$ dead cells was estimated and compared daily. A single WT cell was observed at 72 h whereas the proportion of ICIS-1a$^-$ dead cells dramatically increased after 48 h of RNAi. C. Number of cell divisions per 24 h was estimated for single cells after 24, 48, 72 and 96 h of feeding with bacteria expressing double-stranded ICIS-1a RNA (ICIS-1a$^-$) or with control bacteria (WT). Inhibition of ICIS-1a gene expression significantly reduced the number of cell divisions. D. 48 to 96 h after feeding, cells fed with control bacteria (WT) or with bacteria expressing double-stranded ICIS-1a RNA (ICIS-1a$^-$) were processed for immunostaining of cilia using Axo 49. Control cells presented homogenous distribution of cilia on the cell surface: ¾ of the 4000 cilia were regularly spaced and strictly aligned in longitudinal rows in the cell cortex, while ¼ of the cilia were located in the oral apparatus (arrow heads) where they clustered in a precise pattern (WT). Inhibition of ICIS-1a gene expression led to cells presenting ciliation defects (ICIS-1a$^-$): cells presented a loss of cilia, in particular in the oral apparatus (arrow heads), and some of the remaining cilia were shortened (arrows).

cells (12). However, immunofluorescence experiments using the polyclonal anti-sera did not permit to localize the ICIS-1 protein in primary HNE cells or in human bronchial cell line. As transient transfection of human epithelial primary cells was not efficient, the function of ICIS-1 was assessed in another cellular model. ICIS-1 homologues have been found to be conserved in species which possess motile (*Trypanosoma brucei, Paramecium tetraurelia*) or sensory (*Caenorhabditis elegans*, *Drosophila melanogaster*) cilia (4). So, RNAi experiments were performed using the feeding method for gene silencing in *P. tetraurelia* already described (35). Inhibition of ICIS-1a gene expression led to a reduction in the number of cilia on the cell surface and shortening of remaining cilia. However, duplication of basal bodies was apparently unaffected. These results suggested that ICIS-1 may be involved in cilia stability and/or growth. Moreover, lethality was observed after 48 h of RNAi and may have been induced by the loss of cilia in the oral apparatus: cells possessing an unciliated or partially ciliated oral apparatus cannot easily phagocyte bacteria inducing cell death.

Finally, protein sequence analysis showed that protein ICIS-1 contained a C2 domain, which has been reported to be implicated in calcium-dependent targeting and binding of ligands in numerous proteins (36). C2 domains exert a central role in diverse cellular processes such as membrane fusion, protein transport and signal transduction. Via Ca^{2+} binding, the C2 domain in particular maybe implicated in regulating cilia movement and function (37). Furthermore, the ICIS-1 protein contained a B9 domain. There are two other currently known human proteins containing the conserved B9 domain: EPPB9, and MKS1 (38). But, their biological and molecular properties are so far poorly understood. Recent studies on MKS1 showed a strong expression of the protein in ciliated tissues, especially in the bronchiolar epithelium (39) and predicted that the protein is functionally related to the flagellar (ciliary in humans) basal body proteome (3). Notably, all the three human genes containing a conserved B9 domain are listed among flagellar or cilia-containing species. ICIS-1 was shown to be expressed during MCD and involved in cilia stability. These observations might suggest a cilia-specific role for the B9 domain. However, northern blot analyses showed that the human ICIS-1 was expressed in both ciliated and non-ciliated cell-containing tissues, in contrast to *D. melanogaster* homologues, which were restricted to ciliated cells (4). This suggested that the ICIS-1 protein maybe also necessary for MTOC functions.

Taken together, these results showed that ICIS-1 is a new protein whose function may be related to cilia stability. Characterization of new proteins expressed during ciliated cell differentiation will permit a higher understanding of the ciliogenesis mechanism. Moreover, the unusual genomic localization of this new gene within the TGF-beta1 promoter is an interesting aspect to take into consideration when further assessing regulation during inflammatory and respiratory diseases characterized by defect in cilia formation or function.

6. ACKNOWLEDGMENT

Cecile Ponsard, Marie Skowron-Zwarg, Sandrine Middendorp, and Frederic Tournier contributed equally to this work We acknowledge Prof. Herman (Centre Hospitalier Universitaire Lariboisiere, Paris, France), Prof. Soudan (Hôpital La Pitié Salpêtriere, Paris, France) and their medical staff for nasal turbinates, Chantal Le Mentec for RNAi constructs, Philippe Bastin, Sabrina Absalon and Anne-Marie Tassin for critical discussion and Claude Celati for technical assistance.

7. REFERENCES

1. Dirksen, E. R.: Centriole and basal body formation during ciliogenesis revisited. *Biol Cell*, 72, 31-8 (1991)
2. Dutcher, S. K.: Purification of basal bodies and basal body complexes from Chlamydomonas reinhardtii. *Methods Cell Biol*, 47, 323-34 (1995)
3. Li, J. B., J. M. Gerdes, C. J. Haycraft, Y. Fan, T. M. Teslovich, H. May-Simera, H. Li, O. E. Blacque, L. Li, C. C. Leitch, R. A. Lewis, J. S. Green, P. S. Parfrey, M. R. Leroux, W. S. Davidson, P. L. Beales, L. M. Guay-Woodford, B. K. Yoder, G. D. Stormo, N. Katsanis & S. K. Dutcher: Comparative genomics identifies a flagellar and basal body proteome that includes the BBS5 human disease gene. *Cell*, 117, 541-52 (2004)
4. Avidor-Reiss, T., A. M. Maer, E. Koundakjian, A. Polyanovsky, T. Keil, S. Subramaniam & C. S. Zuker: Decoding cilia function: defining specialized genes required for compartmentalized cilia biogenesis. *Cell*, 117, 527-39 (2004)
5. Ostrowski, L. E., K. Blackburn, K. M. Radde, M. B. Moyer, D. M. Schlatzer, A. Moseley & R. C. Boucher: A proteomic analysis of human cilia: identification of novel components. *Mol Cell Proteomics*, 1, 451-65 (2002)
6. Andersen, J. S., C. J. Wilkinson, T. Mayor, P. Mortensen, E. A. Nigg & M. Mann: Proteomic characterization of the human centrosome by protein correlation profiling. *Nature*, 426, 570-4 (2003)
7. Laoukili, J., E. Perret, S. Middendorp, O. Houcine, C. Guennou, F. Marano, M. Bornens & F. Tournier: Differential expression and cellular distribution of centrin isoforms during human ciliated cell differentiation in vitro. *J Cell Sci*, 113 (Pt 8), 1355-64 (2000)
8. Eley, L., L. M. Yates & J. A. Goodship: Cilia and disease. *Curr Opin Genet Dev*, 15, 308-14 (2005)
9. Wills-Karp, M.: Interleukin-13 in asthma pathogenesis. *Immunol Rev*, 202, 175-90 (2004)
10. Cohn, L.: Mucus in chronic airway diseases: sorting out the sticky details. *J Clin Invest*, 116, 306-8 (2006)
11. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang & J. A. Elias: Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest*, 103, 779-88 (1999)
12. Laoukili, J., E. Perret, T. Willems, A. Minty, E. Parthoens, O. Houcine, A. Coste, M. Jorissen, F. Marano, D. Caput & F. Tournier: IL-13 alters mucociliary differentiation and ciliary beating of human respiratory epithelial cells. *J Clin Invest*, 108, 1817-24 (2001)

13. Tyner, J. W., E. Y. Kim, K. Ide, M. R. Pelletier, W. T. Roswit, J. D. Morton, J. T. Battaile, A. C. Patel, G. A. Patterson, M. Castro, M. S. Spoor, Y. You, S. L. Brody & M. J. Holtzman: Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 transdifferentiation signals. *J Clin Invest*, 116, 309-21 (2006)
14. Wolff, A., B. de Nechaud, D. Chillet, H. Mazarguil, E. Desbruyeres, S. Audebert, B. Edde, F. Gros & P. Denoulet: Distribution of glutamylated alpha and beta-tubulin in mouse tissues using a specific monoclonal antibody, GT335. *Eur J Cell Biol*, 59, 425-32 (1992)
15. Chomczynski, P. & N. Sacchi: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, 162, 156-9 (1987)
16. Szabo, A., C. M. Perou, M. Karaca, L. Perreard, J. F. Quackenbush & P. S. Bernard: Statistical modeling for selecting housekeeper genes. *Genome Biol*, 5, R59 (2004)
17. Sonneborn, T. M.: *Paramecium aurelia*. In: Handbook of Genetics. Ed: R. King. Plenum Publishing Corp., New York (1974)
18. Timmons, L. & A. Fire: Specific interference by ingested dsRNA. *Nature*, 395, 854 (1998)
19. Timmons, L., D. L. Court & A. Fire: Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene*, 263, 103-12 (2001)
20. Dupuis-Williams, P., A. Fleury-Aubusson, N. G. de Loubresse, H. Geoffroy, L. Vayssie, A. Galvani, A. Espigat & J. Rossier: Functional role of epsilon-tubulin in the assembly of the centriolar microtubule scaffold. *J Cell Biol*, 158, 1183-93 (2002)
21. Levilliers, N., A. Fleury & A. M. Hill: Monoclonal and polyclonal antibodies detect a new type of post-translational modification of axonemal tubulin. *J Cell Sci*, 108 (Pt 9), 3013-28 (1995)
22. Rizo, J. & T. C. Sudhof: Mechanics of membrane fusion. *Nat Struct Biol*, 5, 839-42 (1998)
23. Lehner, B. & C. M. Sanderson: A protein interaction framework for human mRNA degradation. *Genome Res*, 14, 1315-23 (2004)
24. Million, K., F. Tournier, O. Houcine, P. Ancian, U. Reichert & F. Marano: Effects of retinoic acid receptor-selective agonists on human nasal epithelial cell differentiation. *Am J Respir Cell Mol Biol*, 25, 744-50 (2001)
25. Ruiz, F., N. Garreau de Loubresse, C. Klotz, J. Beisson & F. Koll: Centrin deficiency in paramecium affects the geometry of basal-body duplication. *Curr Biol*, 15, 2097-106 (2005)
26. Ruiz, F., J. Beisson, J. Rossier & P. Dupuis-Williams: Basal body duplication in Paramecium requires gamma-tubulin. *Curr Biol*, 9, 43-6 (1999)
27. Makalowska, I., C. F. Lin & W. Makalowski: Overlapping genes in vertebrate genomes. *Comput Biol Chem*, 29, 1-12 (2005)
28. Sandler, N. G., M. M. Mentink-Kane, A. W. Cheever & T. A. Wynn: Global gene expression profiles during acute pathogen-induced pulmonary inflammation reveal divergent roles for Th1 and Th2 responses in tissue repair. *J Immunol*, 171, 3655-67 (2003)
29. Massague, J. & D. Wotton: Transcriptional control by the TGF-beta/Smad signaling system. *Embo J*, 19, 1745-54 (2000)
30. Varga, J.: Scleroderma and Smads: dysfunctional Smad family dynamics culminating in fibrosis. *Arthritis Rheum*, 46, 1703-13 (2002)
31. Lee, C. G., R. J. Homer, Z. Zhu, S. Lanone, X. Wang, V. Koteliansky, J. M. Shipley, P. Gotwals, P. Noble, Q. Chen, R. M. Senior & J. A. Elias: Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta (1). *J Exp Med*, 194, 809-21 (2001)
32. Redington, A. E., J. Madden, A. J. Frew, R. Djukanovic, W. R. Roche, S. T. Holgate & P. H. Howarth: Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med*, 156, 642-7 (1997)
33. Moller, G. M., S. E. Overbeek, C. G. Van Helden-Meeuwsen, J. M. Van Haarst, E. P. Prens, P. G. Mulder, D. S. Postma & H. C. Hoogsteden: Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled corticosteroids. *Clin Exp Allergy*, 26, 517-24 (1996)
34. Wenzel, S. E., J. B. Trudeau, S. Barnes, X. Zhou, M. Cundall, J. Y. Westcott, K. McCord & H. W. Chu: TGF-beta and IL-13 synergistically increase eotaxin-1 production in human airway fibroblasts. *J Immunol*, 169, 4613-9 (2002)
35. Galvani, A. & L. Sperling: RNA interference by feeding in Paramecium. *Trends Genet*, 18, 11-2 (2002)
36. Nalefski, E. A. & J. J. Falke: The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci*, 5, 2375-90 (1996)
37. Tamm, S.: Ca2+ channels and signalling in cilia and flagella. *Trends Cell Biol*, 4, 305-10 (1994)
38. Kyttala, M., J. Tallila, R. Salonen, O. Kopra, N. Kohlschmidt, P. Paavola-Sakki, L. Peltonen & M. Kestila: MKS1, encoding a component of the flagellar apparatus basal body proteome, is mutated in Meckel syndrome. *Nat Genet*, 38, 155-7 (2006)
39. Katsanis, N.: Ciliary proteins and exencephaly. *Nat Genet*, 38, 135-6 (2006)

Key words: Cilia, Ciliogenesis, Human Nasal Epithelial Cells, Mucociliary Differentiation, Paramecium, ICIS

Send correspondence to: Dr Sandrine Middendorp, Laboratoire de Cytophysiologie et Toxicologie Cellulaire, Université Paris 7, Tour 53-54, 3eme étage, case 7073, 2 place Jussieu, 75251 Paris cedex 05, France, Tel.: 33-1-44 27-60-72, Fax: 33-1-44-27-69-99, E-mail: middendorp@paris7.jussieu.fr

http://www.bioscience.org/current/vol12.htm