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Nd6p, a Novel Protein with RCC1-Like Domains Involved in Exocytosis in *Paramecium tetraurelia*

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In *Paramecium tetraurelia*, the regulated secretory pathway of dense core granules called trichocysts can be altered by mutation and genetically studied. Seventeen nondischarge (ND) genes controlling exocytosis have already been identified by a genetic approach. The site of action of the studied mutations is one of the three compartments, the cytosol, trichocyst, or plasma membrane. The only ND genes cloned to date correspond to mutants affected in the cytosol or in the trichocyst compartment. In this work, we investigated a representative of the third compartment, the plasma membrane, by cloning the *ND6* gene. This gene encodes a 1,925-amino-acid protein containing two domains homologous to the regulator of chromosome condensation 1 (RCC1). In parallel, 10 new alleles of the *ND6* gene were isolated. Nine of the 12 available mutations mapped in the RCC1-like domains, showing their importance for the Nd6 protein (Nd6p) function. The RCC1 protein is well known for its guanine exchange factor activity towards the small GTPase Ran but also for its involvement in membrane fusion during nuclear envelope assembly. Other proteins with RCC1-like domains are also involved in intracellular membrane fusion, but none has been described yet as involved in exocytosis. The case of Nd6p is thus the first report of such a protein with a documented role in exocytosis.

In the secretory pathway, the last step is exocytosis, which consists of the fusion of the membrane of secretory vesicles with the plasma membrane and allows the release of the vesicular contents into the extracellular medium. In many cells, secretion is regulated by physiological effectors and the level of the regulation stands at different steps in the secretory process. Exocytotic membrane fusion involves well-studied machinery of ubiquitous membrane and soluble proteins, the SNARE complex, and the exocyst (30). The function of this machinery can be regulated by other proteins that are sensitive to external conditions, such as the presence of secretagogues, through signal transduction across the plasma membrane.

A cell model such as *Paramecium tetraurelia* is useful in understanding the last step of exocytotic membrane fusion. On the one hand, the regulation of secretion bears on the very last step of membrane fusion, exocytosis, disconnected from previous steps of biogenesis and docking within the secretory pathway. On the other hand, a genetic approach as well as gene inactivation by RNA interference (RNAi) is an efficient tool currently used in functional studies. This regulated secretory pathway concerns dense core granules of *Paramecium tetraurelia*, trichocysts, which are supposed to be defensive organelles against predators (34). About 1,000 trichocysts are docked under the plasma membrane in a prefusion state, awaiting an external stimulus that triggers massive and synchronous exocytosis within milliseconds (Fig. 1) (27). The docking sites consist of microdomains, at predetermined locations regularly interspersed with cilia along the ciliary rows, recognizable by the presence of a "rosette" of intramembranous particles and a fibrous "connecting material" underneath (7, 39). Trichocyst exocytotic membrane fusion in response to stimulation involves SNARE machinery, as attested by the essential role of an *N*-ethylmaleimide-sensitive factor (15).

A genetic approach revealed nondischarge (ND) genes encoding novel proteins likely involved in exocytotic regulation. These ND mutants have abnormal docking sites: they lack both rosettes and connecting material (7, 28, 39, 41). Physiological analyses involving the transfer of trichocysts from cell to cell by microinjection permit the assignment of a site of action to each mutation (5, 28). ND mutations have been shown to alter three possible compartments, the cytosol (*nd9-1*, *nd16-2*), the trichocyst compartment (*nd2-1*, *nd7-1*, *nd126-1*, *nd169*-1), and the plasma membrane (*nd3-5*, *nd6-1*), as reviewed by Vayssié et al. (59). Previous cloning experiments concerned mutants altering two of these compartments, the cytosol and the trichocyst. The cytosolic Nd9 protein (Nd9p) possesses *Armadillo*-like repeats similar to those found in the yeast protein Vac8, involved in vacuolar fusion, suggesting analogous fusion mechanisms in the two systems (13). The other ND proteins, associated with the trichocyst compartment (Nd2p, Nd7p, Nd22p, and Nd169p), present several cysteine-rich epidermal growth factor and plexin, semaphorin, and integrin (PSI) motifs normally found in extracellular receptors. These observations led to a model in which the assembly of the microdomains at the docking sites would result from a cross-talk between the vesicle and the plasma membrane mediated by intravesicular protein-to-protein interactions involving epidermal growth factor and PSI domains (14).

In this work, we were interested in cloning by functional complementation and studying the *ND6* gene, whose site of action lies in the plasma membrane. We also undertook a new mutagenesis, which led to the discovery of 10 new *ND6* alleles. The sequence of the *ND6* gene indicates that this is a novel gene encoding a protein whose N-terminal half displays two

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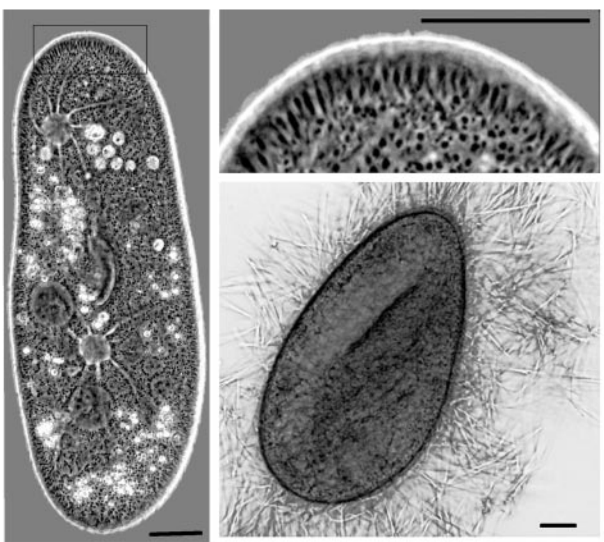


FIG. 1. Localization and secretion of trichocysts: the dense core granules of *Paramecium*. (Left) Phase-contrast image of a living wild-type *Paramecium*. (Top right) Enlargement of part of this cell showing docked trichocysts under the plasma membrane. (Bottom right) Phase-contrast image of a fixed cell after stimulation of exocytosis by a fixing secretagogue (tannic acid, 0.5%). The contents of the secretory granules expand in contact with external media, allowing easy observation of the secretion. Scale bars, 15 μm.

conserved domains found in several proteins, notably the regulator of chromosome condensation 1 (RCC1) protein. Up to now, about 20 proteins with these RCC1-like domains (RLDs) have been described, and 10 of them are cytoplasmic proteins associated with membranous structures; for example, Alsin is a cytoplasmic protein associated with endosomes (38), HERC1 localizes both in cytosol and in the Golgi apparatus (45), and Highwire localizes at presynaptic boutons (61). Two RLD-containing proteins are nuclear and cytoplasmic (Nek9 [44] and DelGEF [58]), and two are only nuclear (RCC1 and TD-60 [8, 35]). Four RLD-containing proteins carry a guanine exchange factor (GEF) activity: RCC1 (42), HERC1 (46), PRAF (23), and Claret (31). RCC1-like domains are also found in proteins involved or suspected of having a role in vesicular membrane fusion. Nd6p, a protein essential for membrane fusion and its regulation during exocytosis, thus appears as a new type of RLD-containing protein that is necessary for the last step of exocytosis.

MATERIALS AND METHODS

Strains and culture conditions. The wild-type strain used was *Paramecium tetraurelia* stock d4-2, derived from stock 51 (52), and the ND mutant lines used in genetic analyses are listed in Table 1. Cells were grown at 27°C in grass infusion (Bio Herbe de Blé [L'arbre de vie, Luçay Le Male, France] or wheat grass powder [Pines International, Lawrence, Kansas]), bacterized with *Klebsiella pneumoniae* the day before use, and supplemented with 0.4 μg/ml β-sitosterol (51).

Monitoring exocytosis. Trichocyst exocytosis capacity was tested using a saturated picric acid solution as a fixing secretagogue. Discharged trichocysts remain clustered around the cell surface (Fig. 1) and can easily be visualized under a dark-field light microscope with a 10× objective. Cells transformed with the TMP1b-green fluorescent protein (GFP) fusion protein (62) with fluorescent trichocysts are triggered with the vital secretagogue aminoethyldextran (AED), and the fact that trichocysts are discharged or retained in the cell is monitored by fluorescence microscopy of living cells or after treatment with an AED-paraformaldehyde-fixing secretagogue solution (see Fig. 5).

Mutagenesis. Mutagenesis procedures were performed as previously described (11, 52). A culture of preautogamous wild-type paramecia of mating type VIII (500 cells per ml) was exposed to UV light (400 J/m^2) and then kept in the dark to avoid DNA repair for 2 days until autogamy occurred. Afterwards, about 40,000 cells were distributed into 100 96-well plates at a mean density of 4 cells per well. Cells were replicated daily three times from the second day after isolation to permit expression of the phenotype and twice at 35°C to look for thermosensitive mutants. Exocytotic capacity was monitored by transferring cells from plates after 2 days of growth into picric acid-containing plates and observation under a stereomicroscope with dark-field illumination. Wells containing cells unable to perform exocytosis were subcloned, and mutant clones were isolated. The tests of allelism among the trichocyst mutants were performed as previously described (12). For the mutant lines isolated, a systematic generation of clones of the complementary mating type VII was performed by screening the rare selfing pairs that occur in cultures of mating type VIII to allow crosses between the cell lines isolated from the mutagenesis.

***ND6* gene cloning.** The *ND6* gene was cloned by functional complementation of the *nd6-1* mutant, by microinjection of DNA into the macronuclei of mutant cells, using a sib selection procedure and DNA prepared from an indexed library of *Paramecium* macronuclear DNA as previously described (25).

Genomic DNA extraction. Total *Paramecium* DNA was prepared from log-phase-culture cells using DNAzol reagent (GIBCO Life Technologies, Paisley, United Kingdom) according to the method recommended by the supplier.

Sequencing and sequence analysis. Random in vitro transposition was performed on the 35I7 insert using the Genome Priming System (Biolabs) in order to provide sequencing templates with internal primer sites. The transposition reaction was carried out using Transprimer-1 (Kan^r) according to the manufacturer's instructions. The clones obtained were sequenced with an ABI 310 sequencer (Perkin Elmer, Foster City, CA) using the BigDye primer cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA). The 12 mutant alleles of the *ND6* gene were then sequenced.

Initial characterization of the DNA and protein sequences was performed with the DNA Strider program (32). The peptide sequence was deduced from the DNA sequence using the ciliate genetic code, where UAA and UAG encode glutamine instead of a stop codon.

Homology searches were performed with the BLAST program (2). Motifs contained in the protein were detected using Prosite (10). Preliminary sequence data on *Tetrahymena thermophila* were obtained from the Institute for Genomic Research website at http://www.tigr.org.

Gene silencing by feeding. Sequences of interest were amplified by PCR and cloned into the L4440 feeding vector between two T7 promoters (54). The *ND6* gene amplification primers were P1 (TTAAACCTGGTATGAAATTACAAGT GAATAATCGAATTAATCC) and P2 (TTAAACCAGGTCAGAGAGTCTTC AAAGTCTTGATGTTTTCC). The resulting constructs were used for the transformation of HT115, an RNase III-deficient strain of *Escherichia coli* with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 polymerase (53). Wild-type paramecia were incubated in double-stranded-RNA-expressing bacteria, as previously described (16). Phenotypes were screened after 24 to 48 h of feeding, and the experiment could be extended for 1 week by daily replication in freshly induced feeding bacteria. Negative controls used HT115 carrying the L4440 plasmid without insertion.

Nd6-GFP fusion constructs. Fusion constructs were made using two distinct plasmids: pPXV (21), in which the GFP synthetic sequence designed by E. Meyer and J. Cohen (unpublished) was introduced (cloning at the 3′ extremity of the GFP), and the pPXV plasmid, with the GFP open reading frame (ORF) designed for expression in *Paramecium* previously described (20) (cloning at the 5′ extremity of the GFP).

The Nd6 ORF was cloned in pPXV-GFP either 5′ from the GFP into the SpeI and XhoI sites using amplification products obtained with primers P3 (TTAAA CTAGTATGAAATTACAAGTGAATAATCGAATTAATCC) and P4 (TTAA CTCGAGTCCACAACGAGTAAACTGTTAGAATTTGTGTC) or 3′ from the GFP into the KpnI site using amplification products obtained with primers P5 (TTAAGGTACCTCACAACGAGTAAACTGTTAGAATTTGTGTC) and P6 (TTAAGGTACCGGAGGAGGAAAATTACAAGTGAATAATCGAATT AATCC).

Labeling of trichocysts was obtained using the TMP1-GFP fusion plasmid from Wassmer et al. (62).

Transformation with GFP fusion constructs. Transformation of *Paramecium* is obtained by injecting a plasmid of interest into the macronucleus of the cell (18). Microinjection of filtered and concentrated plasmid DNA was made under an inverted Nikon phase-contrast microscope, using a Narishige micromanipu-

TABLE 1. Phenotypic characteristics of the *Paramecium* ND mutants[a]

Gene	Allele (reference[s])	Cloning (reference[s])	RNAi (reference[s])	Site of action of the mutation (reference[s])
ND2	*nd2-1* (4, 9, 14, 39, 52)	Yes (9)	Nondischarge (14, 47)	Trichocyst (3)
	nd2-2 (3, 14)			
	nd2-3 (14)			
	nd2-4 (14)			
ND3	*nd3-2* (12, 40)			Plasma membrane (9)
	nd3-3 (ts) (12, 40)			
	nd3-4 (36, 40)			
	nd3-5 (9)			Plasma membrane (9)
ND6	*nd6-1* (9, 12, 27, 40)	Yes	Nondischarge	Plasma membrane (27, 40)
	nd6-3 (9, 12)			
	nd6-5			
	nd6-8			
	nd6-10			
	nd6-12			
	nd6-13			
	nd6-20			
	nd6-21			
	nd6-41			
	nd6-42 (TS)			
	nd6-45			
ND7	*nd7-1* (6, 40)	Yes (50)	Nondischarge (47)	Trichocyst (27, 41)
ND9	*nd9-1* (TS)		Nondischarge (9)	Cytosol (3, 41)
	nd9-2 (TS) (9, 12)			
	nd9-3 (9, 12)	Yes (17)		Cytosol (9)
ND11	***nd11-11***			
	nd11-17			
	nd11-19			
ND12	*nd12-1* (TS) (12)			
ND16	*nd16-1* (TS) (12)			
	nd16-2 (TS) (9)			Cytosol (9)
	nd16-3 (TS) (58)			
	nd16-18			
	nd16-19			
	nd16-24			
ND17	*nd17-1* (TS) (12)			
ND18	*nd18-1* (TS) (9)			
ND19	*nd19-1* (58)			
ND20	*nd20-1* (TS) (37)			
ND21	*nd21-1* (TS) (58)			
	nd21-29 (TS)			
ND22		Yes (14)	Nondischarge (14)	
ND26	***nd26-26*** (TS)			
	nd26-32 (TS)			
ND28	***nd28-1*** (TS)			
ND39	***nd39-1*** (TS)			
ND49	***nd49-1*** (TS)			
ND126	*nd126-1* (9, 36)			
	nd126-2 (6, 9, 36)			Trichocyst (9)

Continued on following page

TABLE 1—*Continued*

Gene	Allele (reference[s])	Cloning (reference[s])	RNAi (reference[s])	Site of action of the mutation (reference[s])
ND146	*nd146-1* (9, 27, 37) *nd146-2* (9, 27, 37)			
ND169	*nd169-1* (9, 14, 37)	Yes (14)	Nondischarge (14)	Trichocyst (9)
ND203	*nd203-1* (9, 27, 37)			
CAM1	*cam1-1* (TS) (24, 49)	Yes (24, 49)		

[a] The table lists all the ND genes known so far, including the ones obtained in the present study (in bold). Thermosensitive-alleles are labeled by TS. No mutant allele exists for the *ND22* gene, found by homology search in the genome with *ND2* (14). The site of action of the mutation (cytosol, cortex, or trichocyst) was determined by microinjection and conjugation experiments (28). The five new genes are *ND11* (three alleles), *ND26* (two thermosensitive alleles), *ND28*, *ND29*, and *ND49* (one thermosensitive allele each). Two new alleles were found for *ND2* (14). Three nonthermosensitive alleles were discovered for the *ND16* gene, and one thermosensitive allele was found for the *ND21* gene. Finally, for the *ND6* gene, for which 2 alleles were already known, 10 new alleles, including a thermosensitive one (*nd6-42*), were obtained. New alleles are in bold. All together, the number of *ND* genes now identified amounts to 23, with a total of 54 alleles. The system, however, still seems far from saturation since 5 new ND genes have been discovered and 11 genes still have a unique allele.

lation device and an Eppendorf air pressure microinjector. Cells clonally derived from microinjected wild-type cells, after 24 or 48 h of growth, were either submitted to the picric acid test to monitor their exocytotic capacity or examined under a Zeiss epifluorescence microscope equipped with a Roper Coolsnap-CF intensifying camera using GFP filters. Images were processed using Metamorph software (Universal Imaging).

RESULTS

The *ND6* gene was genetically identified by the isolation of three mutants, the *nd6-1* (52), *nd6-2*, formerly *nd163* (9), and *nd6-3* (59) mutants. The secretory properties altered by the *nd6-1* mutation have been extensively studied by Lefort-Tran et al. (28). Like other ND mutants, the *nd6-1* mutant is unable to respond to an external stimulus by trichocyst discharge, although trichocysts are normally docked in close apposition to the plasma membrane as in wild-type cells. Ultrastructural examination showed that the mutants lack two types of arrays, rosettes of intramembranous particles and fibrous material connecting the trichocyst and plasma membrane. The transfer of cytoplasm by microinjection showed that the site of action of the *ND6* gene is the plasma membrane (28). This was confirmed by experiments showing that the mutational defect could be repaired through lateral diffusion during cell-to-cell contact that occurs during conjugation with wild-type cells (28).

Cloning *ND6* by functional complementation. Using a previously described method (21, 50) and the indexed library prepared in the laboratory (25), a rescuing activity of the exocytotic deficiency of the *nd6-1* mutant was identified in the plasmid p35I7. Sequencing the 9,241-bp insert of this plasmid revealed the presence of two ORFs of 1,677 bp and 5,778 bp. The *nd6-1* mutant rescuing activity was identified as carried by the 5,778-bp ORF. Indeed, among the plasmids carrying transposons used for sequencing (see Materials and Methods), only those disrupting the 5,778-bp ORF failed to rescue the *nd6-1* mutant. In addition, subcloning this ORF alone allowed rescue of the mutant. The presence of mutations in the *nd6* mutants (see below) confirms that this ORF actually corresponds to the *ND6* gene and not a multicopy suppressor. Southern blot analysis, together with a BLAST search of the recently sequenced *Paramecium* genome, indicated that this gene is unique. An mRNA of approximately 6 kb could be detected by Northern blot analysis (data not shown), showing that the gene is transcribed. The detected ORF corresponding to the *ND6* gene has no predictable intron and encodes a 1,925-amino-acid protein.

The Nd6 peptide presents two RCC1-like domains. Homology searches of *ND6* by BLAST revealed no true full-length homologues in other species, except in the recently sequenced genome of *Tetrahymena* (http://www.tigr.org) in which two homologues placed in tandem can be detected (although they appear as a single ORF in the automatic annotation on the website http://www.ciliate.org [annotation number, 44.m00200]). Nd6p of *Paramecium* shares 27% identity and 44% similarity with the first homologue of *Tetrahymena* and 25% identity and 42% similarity with the second homologue (Fig. 2). The *Tetrahymena* Nd6p homologues share 33% identity and 52% similarity with each other.

The N-terminal half of *Paramecium* as well as *Tetrahymena* Nd6p displays conserved RLD motifs, first identified in the RCC1 protein (RCC1p). RCC1p, conserved from yeast to human, is a GDP exchange factor (GEF) for the small GTPase Ran (1, 26), which plays a major role in nucleocytoplasmic transport. RLDs are composed of seven imperfect repetitions of 45 to 70 or more amino acids, which form seven-bladed propellers, each blade containing four antiparallel β-sheets, thus the name of β-propellers (43). The primary structure of Nd6p, as well as of both homologous genes in *Tetrahymena*, contains two adjacent RLDs of seven repeats each, separated by 25 to 60 amino acids (Fig. 3). This suggests that the 14

FIG. 2. Alignment of Nd6 proteins of *Paramecium* and *Tetrahymena*. Alignment of the Nd6 homologues of *Tetrahymena thermophila* (Nd6_Tt1 and Nd6_Tt2) with Nd6 of *Paramecium tetraurelia* (Nd6_Pt). This alignment was realized using the MultAlin program (http://prodes.toulouse.inra.fr/multalin/multalin.html). Nd6_Pt shares 27% identity and 44% similarity with Nd6_Tt1 and 25% identity and 42% similarity with Nd6_Tt2. Nd6_Tt1 and Nd6_Tt2 share 33% identity and 52% similarity with each other.

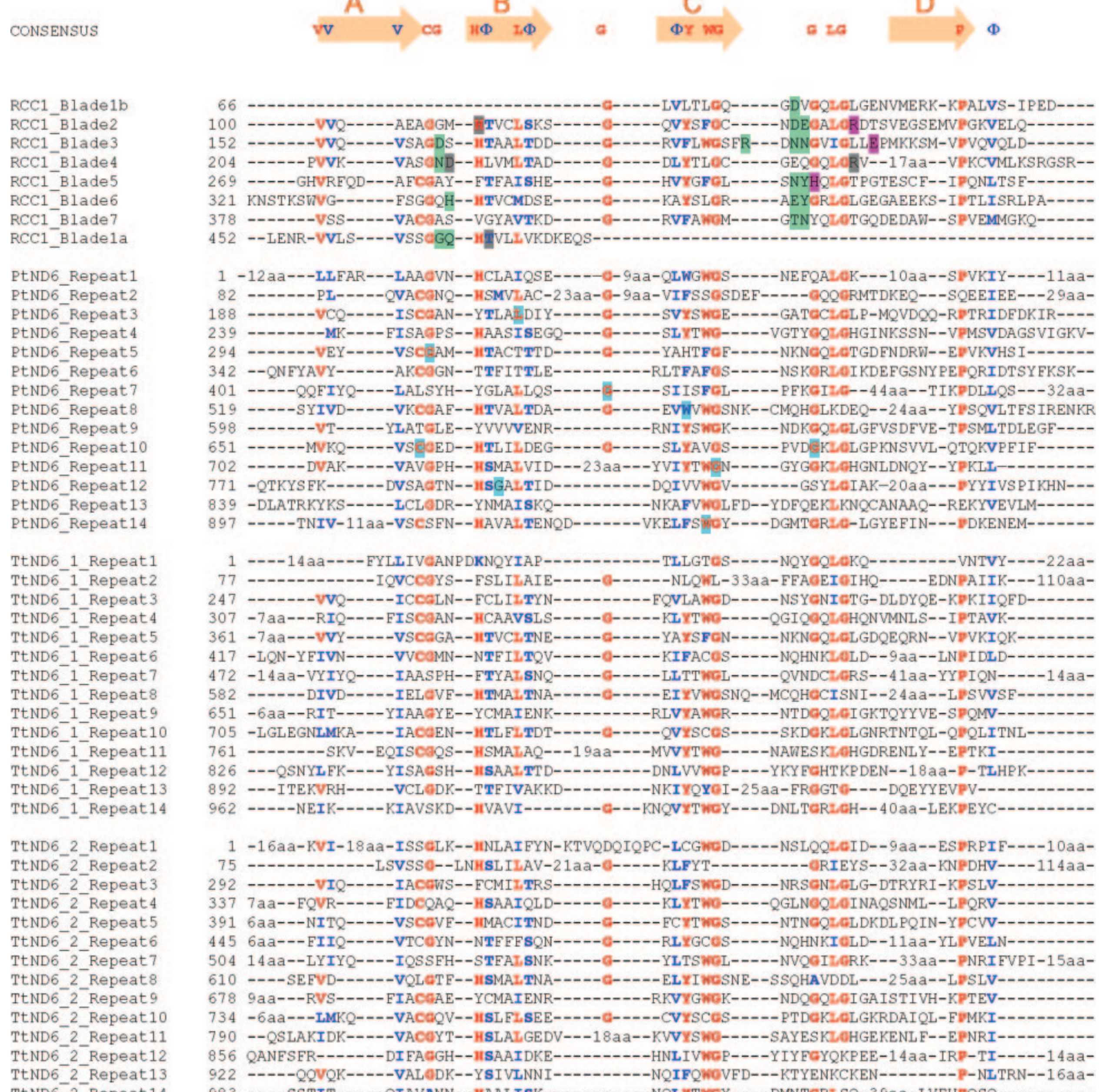


FIG. 3. RCC1 repeats of Nd6p. Alignment of the RCC1 repeats of the human RCC1 protein and the two RLDs of *Paramecium tetraurelia* (Pt) and *Tetrahymena thermophila* (Tt) Nd6 proteins. The first line (consensus) represents the simplified structure of RCC1 repeats, where arrows represent β-sheets and A, B, C, and D represent the common names of these β-sheets. Φ, hydrophobic residue. Identities are shown in red, and similarities are shown in blue. In the RCC1 sequence, residues implicated in the Ran-RCC1 interaction have a green background and residues provoking a decrease of k_{cat} (the overall catalytic rate of the enzyme) and K_m (the concentration of substrate at which the rate of the reaction is one-half of the maximal rate) when mutated are shown, respectively, with a magenta and a gray background (42, 43). In the Nd6p sequence, the positions of the mutations are shown with a cyan background. All these mutations affect conserved residues, suggesting that these amino acids are essential for the structure of the peptide. The residue affected in the *nd6-8* mutant (G680E in repeat 10) is at a position corresponding to a mutation in human RCC1 affecting its affinity for Ran, indicating that this residue in Nd6p could also be essential for interaction with a small GTPase.

β-sheets could adopt a structure of two contiguous β-propellers. Since RCC1p is a GEF like other RLD-containing proteins, such as HERC1, ALS2, etc. (38, 42, 46), Nd6p could also have the GEF activity necessary for its function in exocytosis.

In order to know whether Nd6p is the only RLD-containing protein encoded in the genome, thus being the *Paramecium* RCC1 orthologue, TBLASTN searches on the draft genome sequence using RCC1 proteic sequences were realized. Among 25 potential proteins containing RCC1 repeats detected, at least one protein (accession number CAH03314) seems to be more similar to the human RCC1 protein. This protein, composed exclusively of RCC1 repeats, shows the same properties

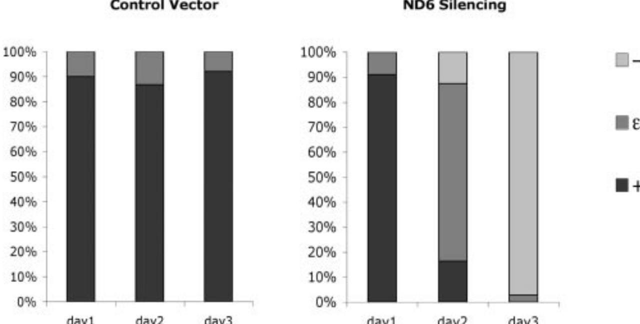
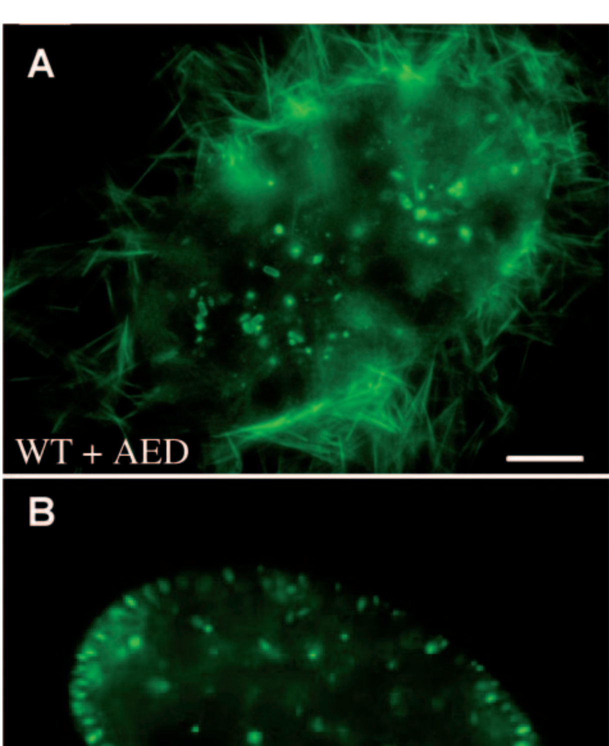


FIG. 4. Effects of gene silencing on exocytosis. The symbols +, ε, and – indicate the mean exocytotic capacities of silenced populations. +, between 500 and 1,000 trichocysts discharged per cell; ε, between 1 and 50 trichocysts discharged; –, no trichocysts discharged. (Left) Paramecia fed with bacteria carrying the L4440 vector without insertion. (Right) *ND6* silencing. One day after feeding paramecia with double-strand *ND6* RNA-producing bacteria, we can see an alteration of the exocytotic capacity compared to that of control cells fed with nontransformed HT115 bacteria (day 2). The phenotype is stronger on day 3.



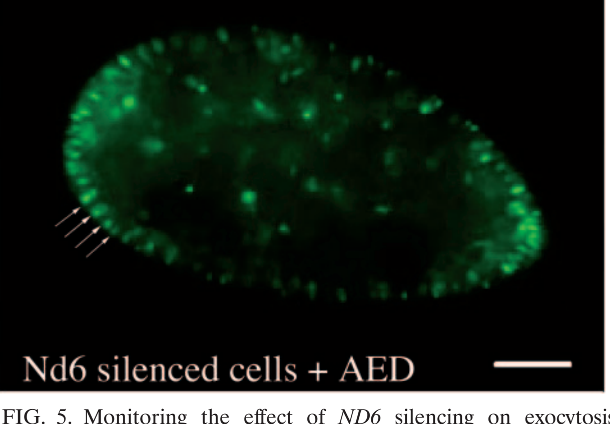


FIG. 5. Monitoring the effect of *ND6* silencing on exocytosis. (A) Exocytosis of a wild-type (WT) cell expressing the trichocyst matrix protein 1 fused to GFP (TMP1-GFP) (62) after treatment with a fixing secretagogue solution (2% paraformaldehyde, 1% AED, 1 mM $CaCl_2$, 10 mM Tris, pH 8.5). (B) The *ND6*-silenced cell is unable to perform exocytosis. The trichocysts, labeled with TMP1-GFP, are blocked inside the cell under the plasma membrane (arrows). Scale bars, 20 μm.

as those of the RCC1 protein: the first blade of the β-propeller mixes two repeats from the N terminus with two repeats from the C terminus, and the region specific for interaction with Ran (42) is present. Thus, Nd6p could be an RLD-containing protein with a function distinct from the nucleocytoplasmic transport activity of RCC1p.

Functional analyses of Nd6p. In *Paramecium*, gene silencing can easily be obtained by feeding wild-type paramecia with bacteria producing double-stranded RNA corresponding to the target sequence (16). The N-terminal half of the *ND6* sequence encoding the RLDs (2,940 bp) was cloned into the L4440 plasmid, between two T7 promoters, and introduced into the *E. coli* strain HT115. These bacteria were then used to silence the *ND6* gene of wild-type paramecia. To improve visualization of the phenotype, we also silenced wild-type cells in which trichocysts were made fluorescent by transformation with the *TMP1* gene fused with GFP (62). Cells fed with bacteria expressing *ND6* double-stranded RNA were subjected to different observations: trichocyst discharge ability, trichocyst biogenesis and attachment to the cortex, cell growth rate, swimming behavior, cell morphology and morphogenesis, and ciliary growth capacity after deciliation. None of the tested phenotypes of *ND6*-silenced cells differed from those of wild-type cells, except in the abolishment of trichocyst discharge capacity, an ND phenotype (Fig. 4). The result was the same whether we used wild-type cells or cells with fluorescently labeled trichocysts (Fig. 5). In agreement with the phenotype of the *nd6* mutants, the silencing observations show that the *ND6* gene is essential for, and the only gene involved in, the final step of trichocyst exocytosis.

In attempts to confirm that the site of action of Nd6p lies in the plasma membrane by direct localization of the protein, we tagged the protein with GFP. The GFP sequence was fused to the *ND6* gene, either at its 5′ or 3′ end, and expressed under the strong promoter of calmodulin in pPXV (21). However, by transformation of the *nd6-3* mutant with these constructs, we never obtained any fluorescence labeling in the cells or rescue of the exocytotic deficiency. Interestingly, transformation of wild-type cells with the same construct not only gave the same absence of fluorescence labeling but also produced a phenocopy of the mutants, with a lack of trichocyst discharge capacity, suggesting that the GFP fusion altered the function of Nd6p and reciprocally that the Nd6p sequence prevented the appearance of GFP fluorescence. We thus postulate that the altered Nd6-GFP fusion protein has a dominant negative effect on the assembly of fusion-competent microdomains.

Ten new alleles in the *ND6* gene obtained by mutagenesis. We carried out a new mutagenesis to increase our collection of mutants. Exocytosis-deficient mutants were screened in the mutagenized population after autogamy by picric acid tests in microtiter plates (see Materials and Methods). Twenty-four mutant lines were isolated through this procedure. Crosses between all the new mutants allowed us to identify nine complementation groups. One representative of each group was crossed with 17 mutants, each one representing already-known ND genes. All together, 24 novel alleles were obtained, among which 8 mapped to five new ND genes and 16 mapped

FIG. 6. Mutations in the *ND6* gene. Overall organization of the 1,925-amino-acid-long Nd6 protein. Gray boxes represent the 14 repeats of the RCC1-like domains, called RLD1 and RLD2. The localization and the nature of the mutations are indicated by the allele numbers.

to previously known ND genes (Table 1). The surprising result of this mutagenesis is that, among the new mutations, 10 mapped to the *ND6* gene, giving a total of 13 mutated alleles for this gene. One allele, *nd6-2*, was lost, and the 12 others were subjected to further characterization.

The majority of the mutations of *ND6* are localized in the region encoding RCC1-like domains. The 12 *ND6* alleles were sequenced, and a single base substitution was found in the *ND6* ORF for each of them (Fig. 6). Interestingly, all mutations, whether they are in the N-terminal RCC1 domains or in the C-terminal tail, give the same loss-of-function phenotype.

Three mutations are nonsense mutations that lead to a truncation of the protein, the *nd6-10*, *nd6-21*, and *nd6-45* mutations. Two mutations affect the C-terminal part of the protein, the *nd6-41* and *nd6-3* mutations, the latter affecting a potential tyrosine kinase phosphorylation site predicted by the Prosite program. The seven other mutations affect residues of the RLD parts of the protein. Three mutations affect amino acids of the first RLD (*nd6-20*, *nd6-13*, and *nd6-42* [TS]), and four affect amino acids of the second RLD (*nd6-1*, *nd6-5*, *nd6-12*, and *nd6-8*). All abolish the function of Nd6p. Among these mutations, four concern conserved residues (*nd6-1*, *nd6-5*, *nd6-13*, and *nd6-20*) which, when mutated in RCC1p, destabilize the β-propeller structure (43). These results suggest that the RLDs of Nd6p really do adopt a β-propeller structure and that this structure is altered in the mutants. Interestingly, equivalents of three of these mutations (*nd6-1*, *nd6-13*, and *nd6-20*) are also found in the retinitis pigmentosa GTPase regulator (RPGR) gene (60).

In addition, the *nd6-8* mutation (G680E) alters a conserved residue between two β-sheets, C and D (Fig. 3), at a position corresponding to a mutation known in the human RCC1 protein to affect the affinity for Ran (42), although not in the loop found in RCC1p specific for interaction with Ran. This suggests not only that this mutation affects a residue essential for the interaction between Nd6p and a putative GTP-binding partner, potentially driving GEF activity, but also that this interaction and this GEF activity are necessary for trichocyst exocytosis.

DISCUSSION

In this work, we have identified the Nd6 protein of *Paramecium*, controlling exocytosis, as a novel protein of 1,925 amino acids containing two RCC1-like domains in its N-terminal half. The phenotype given by the mutations in the *ND6* gene, as well as the results of RNAi and overexpression of GFP fusion experiments, has shown that the only role detected for *ND6* is in the last step of the trichocyst-regulated secretory pathway, exocytosis. The fact that full-length homologues seem to exist in *Tetrahymena* suggests that the homologous function, mucocyst exocytosis, could also be dependent on these genes.

The sequence of 12 *ND6* mutant alleles revealed that most of the mutations affect well-conserved residues, localized in the RLDs. In addition, four of them touch residues for which the mutations in the human RCC1 protein alter the three-dimensional structure (43). This suggests that the N terminus of Nd6p may adopt a structure with two adjacent β-propellers and that the structure may be destabilized in the mutants. The occurrence of such mutations also indicates that Nd6p requires the integrity of both RLDs for its function. From what is known of RLDs, three kinds of information can help in understanding the mode of action of Nd6p: RLDs are the substrate of protein-to-protein interactions necessary for their function (17), they often carry a GEF activity for small GTPases (17), and they may be involved in membrane fusion.

Since the mode of action of RLDs, when it is known, is through protein-to-protein interactions, Nd6 may perform its function by interacting with one or several proteic partners. To find such potential partners, we planned to explore the genome data to find in silico candidates and undertake systematic large-scale RNAi analyses. In a first attempt, we tried to silence two single-copy genes (*P. tetraurelia PDE*δ and *DelGIP*), but no evidence that these genes were involved in exocytosis, and thus that they could interact with Nd6p, was obtained (data not shown). Another approach that may be undertaken is to search interacting proteins by biochemical approaches, using procedures that overcome the code deviation of *Paramecium* to produce the amount of Nd6p necessary for antibody production.

Many RLD-containing proteins bind small GTPases (Table 2): RCC1 and Nek9 (Nercc1) bind Ran (8, 44); TD-60 binds Rac1 (35); ALSIN binds Rac1 and Rab5 (55); HERC1 binds ARF1, Rab3, and Rab5 (46); PRAF binds Rab8 (23); and Claret binds Lightoid (Rab-RP1) (31). Four of them (RCC1, HERC1, PRAF, and Claret) display a GEF activity. The possibility that Nd6p can associate with a small GTPase, which remains to be identified, and carry a GEF activity is supported by the fact that the *nd6-8* mutation (G680E) corresponds to a mutation localized in one of the Ran-binding sites, between two β-sheets of the RCC1p β-propeller, and that abolishes Ran binding.

Some RLD-containing proteins (RCC1, ALSIN, and HERC1) have a well-established function in intracellular vesicle fusion as summarized in Table 2. For example, ALSIN

TABLE 2. Correlation between RLD-containing proteins and membrane fusion defects[a]

Protein	Mutant phenotype and/or function(s) (reference)	Binding to GTPases (reference)
RCC1 RCC1 1 421	Premature chromosome condensation (57) Defects in nuclear pore complex assembly (48) Abnormal nuclear morphology (48) Vesicule accumulation and extended cytoplasmic membrane structures (48)	+ (8) Ran GEF activity (8)
ALSIN RCC1 DH PH MORN VSP9 1 1657	Amyotrophic lateral sclerosis (19) Defects in endosomal trafficking (38) ALS2 promotes neurite outgrowth (56)	+ (38) Rab5, Rac1 GEF activity (38)
HERC1 RCC1 β repeats RCC1 HECT 1 750 3000 4861	Important role in intracellular trafficking in the Golgi apparatus and the cytoplasm (45)	+ (45) ARF1, ARF6, Rab3a, and Rab5a GRF activity (17)
HERC2 RCC1 Cyt B5 RCC1 RCC1 HECT 1 1250 3000 3310 3950 4836	rjs syndrome (runty, jerky, sterile) (29) Role in the secretory trafficking pathways of mainly neurons and sperm cell precursors (17)	NS
RPGR RCC1 1 1020	Retinis pigmentosa (33) Mislocalization of essential membrane proteins (opsin and rhodopsin) (22) Disorganization of newly formed disk membranes at the base of photoreceptor (22)	NS
CLARET RCC1 1 1961	Eye pigment defects (31) Defects in the vesicular pathway of eye pigment granule biogenesis (31)	+ (31) Rab-RP1
ND6 RCC1 RCC1 1 1925	Lack of regulated exocytosis of dense core granules	NS

[a] The figure lists the RLD-containing proteins for which a function in vesicular fusion has been described (RCC1, ALSIN, and HERC1) and the proteins for which an alteration induces defects that could be attributable to membrane fusion defects (HERC2, RPGR, and Claret). The table gives the scheme of each protein, its supposed function(s) or the phenotype observed when it is altered and its associated small GTPase. GRF activity, guanine nucleotide release factor activity; NS, not shown.

plays an important role in endosomal dynamics, including endosome trafficking and fusion by activating Rab5 (38). It has also been observed that the alteration of some proteins containing RCC1 domains (HERC2, RPGR, and Claret) induces defects that could be attributable to membrane fusion defects (Table 2). All these proteins present a modular architecture and have in common only the presence of RCC1 domains and a function in membrane fusion. We can thus envisage that the RCC1 domains are responsible for this role in membrane fusion.

Our results not only allow us to add Nd6p to this list of RLD-containing proteins, a candidate to be involved in membrane fusion, but also suggest a new function for the RLD-containing proteins: the control of the terminal step of regulated exocytosis, the fusion of secretory granules with the plasma membrane.

The mechanism by which RLD-containing proteins can intervene in exocytosis is open. We can anticipate that the mechanism could be compared to the ones by which other GEFs act in the control of membrane fusion. For example, ARF-GEF containing Sec7 domains or Rab-GEF activities associated with multiproteic complexes, such as the exocyst, allow the control of membrane fusion promoted by the SNARE machinery in various donor or target compartments. A similar role in membrane fusion could be imagined for RLD-containing proteins with GEF activity.

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