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Genetic Approach to Regulated Exocytosis Using Functional Complementation in *Paramecium*: Identification of the *ND7* Gene Required for Membrane Fusion

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Paramecium is a unicellular organism that possesses a specialized pathway for regulated secretion that is amenable to genetic studies. Numerous mutations affecting the process have been isolated over the years, among which is a subclass blocking the terminal step of fusion of the secretory granule with the plasma membrane. We report herein the cloning by functional complementation of one such gene, *ND7*. The 506-amino acid polypeptide encoded by *ND7* is predicted to be a type I integral membrane protein with a highly charged cytosolic domain featuring amphipathic and coiled-coil regions. This structure is compatible with the physiological data on the mutant nd7–1 suggesting that the protein is anchored in the membrane of the secretory granule and that it may interact with other proteins. This work presents the first identification by a genetic approach of a novel gene involved in regulated secretion and establishes *Paramecium* as a powerful model system for the genetic dissection of this process.

INTRODUCTION

In regulated secretion, secretory products are stored in specific organelles and released only upon extracellular stimulation, through a still poorly understood mechanism involving a large number of proteins. This pathway is restricted to certain specialized cell types in metazoa (i.e., neuronal, exocrine, and endocrine cells) and is absent from most unicellular organisms such as yeast. Regulated secretion can, however, be studied in the ciliate *Paramecium*, where numerous mutations affecting this process have been obtained. In *Paramecium*, this pathway concerns secretory granules called trichocysts (approximately 1000 per cell) that develop in the cytoplasm, then move to the cell surface, and dock at the plasma membrane. The trichocysts remain attached at their specific docking site until the last step of the secretory pathway, membrane fusion and exocytosis of the contents, is triggered by an external stimulus. As shown in Figure 1, these sites are characterized by well-defined structures visible in electron microscopy: a "rosette" of intramembranous particles in the plasma membrane just above the trichocyst tip and a fibrous "connecting material" that links the trichocyst membrane to the plasma membrane (Plattner *et al.*, 1973; Beisson *et al.*, 1976). The assembly of these structures, therefore, marks the stage at which membrane fusion is naturally "frozen" in *Paramecium*.

Some of the particular features of *Paramecium* regulated secretion are also found in other systems: the cortical granules of oocytes (Gulyas, 1980) and the subpopulation of synaptic vesicles that is ready for membrane fusion in nerve terminals (Pieribone *et al.*, 1995) are docked under the plasma membrane; rosette-like intramembranous particles have been observed above docked synaptic vesicles at the neuromuscular junction (Heuser *et al.*, 1979) and links between secretory granules and plasma membrane have been detected in chromaffin cells (Aunis *et al.*, 1979) and in mast cells (Chandler and Heuser, 1980). However, these links are generally hard to visualize since they cover a small portion of the vesicle area and

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often appear transiently during the stage of membrane fusion. In recent years, a number of interacting proteins involved in vesicle docking, priming, and membrane fusion, such as *N*-ethylnaleimide-sensitive factor (NSF), soluble NSF-attachment (SNAP) and SNAP receptors (SNAREs), have been identified biochemically in mammalian cells and genetically in yeast (Rothman, 1994; Südhof, 1995). However, such protein complexes are also involved in earlier steps of intracellular membrane trafficking common to both regulated and constitutive secretion. Only a few proteins such as synaptotagmin are known to be specific for the control of the response to stimulation in regulated secretion (Bennett and Scheller, 1993).

Genetic analysis of exocytosis in *Paramecium* has already demonstrated that the rosette and the connecting material are involved in membrane fusion (Beisson *et al.*, 1976, 1980; Lefort-Tran *et al.*, 1981; Pouphile *et al.*, 1986). Indeed, because trichocyst exocytosis is not a vital function under laboratory conditions, many secretory mutants have been isolated over the last 20 y by using a simple and sensitive visual assay (Figure 2). Twenty-three recessive nuclear mutations, called *nd* for nondischarge, specifically block exocytotic membrane fusion and map to 13 distinct loci (Cohen and Beisson, 1980; Bonnemain *et al.*, 1992). A mutation in the calmodulin gene *cam1* (Kink *et al.* 1990) also displays a thermosensitive nd phenotype (Kerbœuf *et al.*, 1993). Mutations at most of these loci, including *cam1*, affect the assembly of the connecting material and the rosette (Bonnemain *et al.*, 1992), and a mutation at one locus, *ND12*, abolishes the calcium influx required for exocytosis (Kerbœuf and Cohen, 1990) but not the assembly of these structures (Pouphile *et al.*, 1986). The *Paramecium* system presents an additional unique advantage: the site of action of the products of the *ND* genes can be established by transferring cytoplasm containing a few trichocysts from one cell type to another in appropriate combinations of donor and recipient cells and checking the exocytotic capacity of the injected cells (Aufderheide, 1978; Lefort-Tran *et al.*, 1981). Some ND gene products have thus been localized in the trichocyst compartment (e.g., ND7p), in the cytosol (e.g., ND9p) or in the plasma membrane (e.g., ND6p; Aufderheide, 1978; Beisson *et al.*, 1980; Lefort-Tran *et al.*, 1981).

Recently, a method for cloning *Paramecium* genes by functional complementation has been devised by W.J. Haynes *et al.* (1996). We report herein use of this method to clone the *ND7* gene (deposited in the EMBL database as accession number Y07803). The sequence indicates that it is a novel gene encoding a type I integral membrane protein whose cytoplasmic domain could be involved in protein–protein interactions. The role of this protein in the assembly of the connecting material is discussed.

MATERIALS AND METHODS

Strains

The wild-type strain of *Paramecium tetraurelia* is Stock d4–2, a derivative of Stock 51 (Sonneborn, 1975). The mutant nd7–1 was found associated with another mutation, *cl1* (Sainsard *et al.*, 1974), after UV mutagenesis. *nd7–1* is the only extant allele and is highly penetrant. Indeed, the trichocysts of the mutant, as in other nds, are normally docked in their cortical site under the plasma membrane but cannot undergo exocytosis: fewer than 0.1% of nd7–1 paramecia can discharge 1–10 trichocysts.

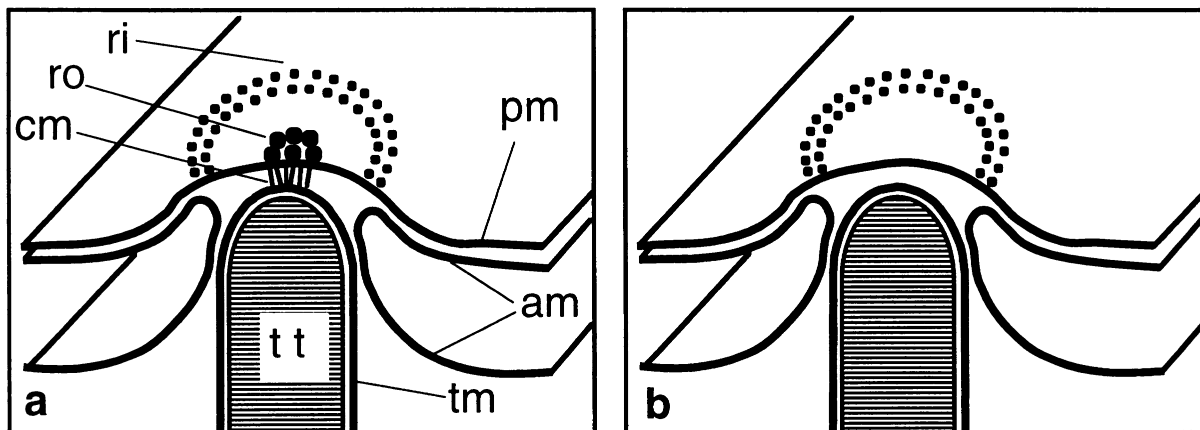

Figure 1. Schematic view of the organization of a trichocyst docking site in wild-type and nd mutants. (a) In the wild-type, a connecting material (cm), visible in transmission electron microscopy, links the trichocyst membrane (tm) to the plasma membrane (pm) at a location where a rosette (ro) of intramembranous particles is observed at the center of a double ring (ri) in freeze-fracture electron microscopy. am, alveolar membranes delineating the subplasmalemmal calcium stores; tt, trichocyst tip. (b) In all nd mutants except nd12, the docking sites lack both rosette and connecting material (Beisson *et al.*, 1976, 1980; Lefort-Tran *et al.*, 1981; Pouphile *et al.*, 1986, Bonnemain *et al.*, 1992). Docking sites of nd7–1 cells are described in freeze-fracture electron microscopy by Lefort-Tran *et al.* (1981) and in transmission electron microscopy by Pouphile *et al.* (1986).

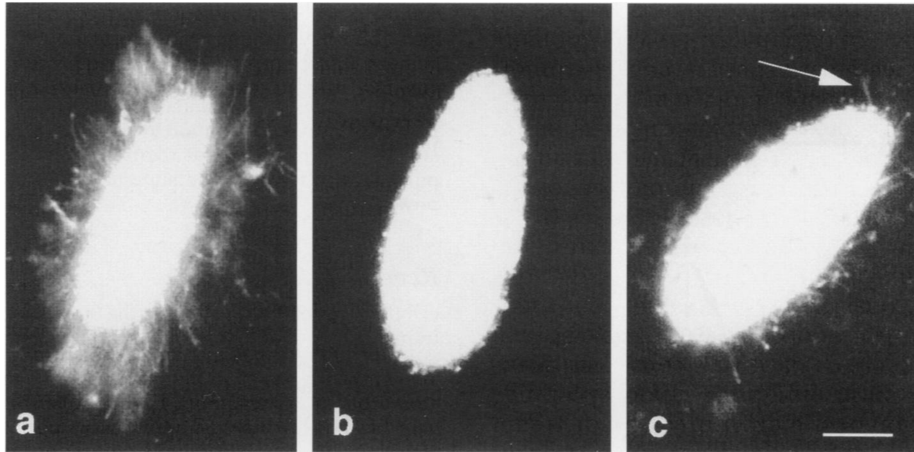

Figure 2. Visual monitoring of exocytosis in *Paramecium* by picric acid treatment. (a) Wild-type cell with approximately 1000 discharged trichocysts visible as small needles regularly distributed all over the cell surface. (b) nd7–1 mutant cell unable to undergo exocytosis. (c) Mutant cell of an intermediate phenotype (nd7–1 cell partially rescued by transformation) showing that even a few discharged trichocysts can be individually detected. The arrow points to one such individual trichocysts. Bar, 20 μm.

Culture Conditions

Cells were grown at 27°C in wheat grass powder (Pines International, Lawrence, KS) infusion, bacterized the day before use with *Klebsiella pneumoniae*, and supplemented with 0.4 μg/ml β-sitosterol, according to Sonneborn (1970).

Monitoring Exocytosis

To visualize individual cells with their own discharged trichocysts, a saturated solution of picric acid is used as a fixing secretagogue. Discharged trichocysts remain attached to the cell so that discharge is easily monitored under dark-field light microscopy with a 10× objective (Figure 2).

Purification of Macronuclear DNA

Ten liters of late logarithmic phase culture (3000 cells/ml) were centrifuged for 1 min at 30 × *g*, and the cell pellet was washed in Dryl's buffer (2 mM sodium citrate, 1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 1.5 mM $CaCl_2$). After a 1-h incubation in Dryl's buffer, cells were harvested, and the pellet was washed twice in 0.25 M sucrose and 10 mM $MgCl_2$ and then homogenized in 1 volume of 0.25 M sucrose, 10 mM $MgCl_2$, 10 mM Tris (pH 7.2), and 0.2% Nonidet P-40. The lysate was centrifuged for 1 min at 100 × *g*. The supernatant containing most of the mitochondria and of the micronuclei (the sole sources of nonmacronuclear DNA; Preer *et al.*, 1992) was discarded. The pellet was solubilized in 8 volumes of 0.5 M EDTA (pH 9), 1% SDS, 1% Sarkosyl, and 1 mg/ml proteinase K and incubated overnight at 55°C. The macronuclear DNA was then purified by two phenol-chloroform and one chloroform extractions and centrifugation on a CsCl gradient.

DNA Digestions and Size Fractionation

The restrictions enzymes *Bcl*I, *Bgl*II, *Eco*RV, *Hin*dIII, *Swa*I, and *Xba*I were chosen because they cut *Paramecium* DNA into fragments between 0.5 and 12 kb long. The digests were either concentrated by precipitation, for rescue experiments, or loaded on a preparative agarose gel, for size fractionation. A gel containing 200 μg of wild-type DNA digested by *Bcl*I was cut into six bands of <0.5 kb, 0.5–1 kb, 1–2 kb, 2–4 kb, 4–8 kb, and >8 kb, and the DNA was extracted from the bands with agarase (Sigma, St. Louis, MO).

DNA Cloning

The 4- to 8-kb fraction from the *Bcl*I digest was cloned into the *Bam*HI-compatible site of the plasmid pBluescript IISK− (Stratagene, La Jolla, CA) with an insert:vector molar ratio of 3:1 after treatment of the vector with shrimp alkaline phosphatase (United States Biochemical, Cleveland, OH). The ligation product was electroporated into XL2-Blue *Escherichia coli* cells. During the sib selection (Figure 3), the complexity of the library was diminished by a factor of 6 at each step. The first 13,500 clones were plated on six Petri dishes, and two copies of the dishes were kept on nitrocellulose replicas. The replica corresponding to a rescuing sublibrary was cut into six pieces. After identification of the nitrocellulose piece containing the rescuing clone, 1296 colonies from this piece of nitrocellulose were streaked on six matrices. The rescuing pool was further divided into smaller and smaller pools until the unique rescuing pool had been identified. In all cases, library colonies were replica plated with nitrocellulose filters to prepare DNA directly from the replicated culture plates, to avoid possible bias during growth in liquid culture.

Purification of DNA from Bacterial Libraries

Library and sublibrary DNAs were prepared from bacteria resuspended from culture plates by lysing cells in 50 mM Tris-HCl (pH 8), 25% sucrose, and 10 mg/ml lysozyme; further incubated in 50 mM Tris-HCl (pH 8), 60 mM EDTA, and 0.1% Triton X-100; and purified by centrifugation on a CsCl gradient.

Microinjection of DNA into the Macronucleus

DNA to be tested for rescuing activity was ethanol precipitated, resolubilized in water at a concentration of 5 mg/ml, and microinjected into the macronucleus of the mutant nd7–1 by using an inverted Nikon phase-contrast microscope, a Narishige micromanipulation device, and air-pressure microinjection. The clonally derived offspring of microinjected cells were tested after 24 h of growth for their exocytotic capacity. Clones harboring at least one cell with more than 10 trichocysts or several cells with at least one trichocyst were scored as rescued.

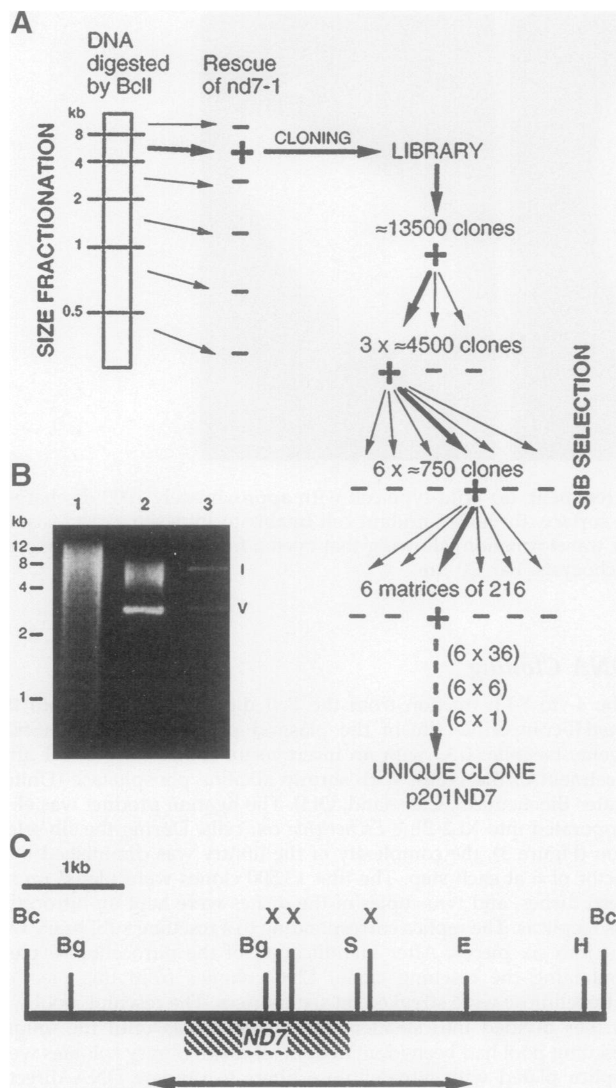

Figure 3. Cloning of $ND7^+$ by functional complementation and sib selection. (a) Wild-type macronuclear DNA digested by *Bcl*I, conferring a rescuing activity on nd7–1 cells, was size-fractionated with preparative gel electrophoresis and six fractions (>8 kb, 4–8 kb, 2–4 kb, 1–2 kb, 0.5–1 kb, and <0.5 kb) were recovered. The fractions were then tested for their ability to rescue *nd7–1*, and the active fraction (4–8 kb) was cloned into pBluescript IISK−. DNA extracted from the resulting 13,500 clone library was able to complement the mutant. Six successive steps of subdividing the library into pools, including the streaking of 1296 clones into six matrices of 216 and testing for nd7–1 rescue were performed to track and isolate the unique $ND7^+$ clone, called p201ND7. (b) Gel illustrating the three main steps in the cloning procedure. Lane 1, total *Bcl*I digest of wild-type DNA; lane 2, DNA of the 13,500 clone library cut by *Bss*HII to separate the inserts (a regular smear between 4 and 8kb) from the vector; lane 3, DNA of p201ND7 cut by *Bss*HII revealing a 5.5-kb insert. I, inserts; V, vector. (c) Restriction map of the 5.5-kb insert. Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RV; H, *Hin*dIII; S, *Swa*I; X, *Xba*I. Hatched bar, open reading frame; double arrow, 3-kb region that has been sequenced.

Purification of Paramecium mRNA

Paramecium mRNAs were purified with the Quick prep mRNA purification kit from Pharmacia-LKB Biotechnology (Piscataway, NJ), with slight modifications of the protocol for adaptation to this cell type. Briefly, 1 l of logarithmic-phase *Paramecium* culture (1500–2000 cells/ml) was harvested and the cell pellet was homogenized in 20 volumes of 6 M guanidine thiocyanate, 0.75% sarkosyl, 37 mM sodium citrate (pH 6.8), and 0.1 M 2-mercaptoethanol. Proteins were precipitated by a 1:4 dilution in H_2O and removed by centrifugation at 5500 × *g* for 10 min at room temperature. Poly$(A)^+$ RNA was purified directly from the supernatant by oligo(dT) chromatography.

Reverse Transcription

cDNAs were obtained by using the Superscript II RNase H^- reverse transcriptase (Life Technologies, Gaithersburg, MD) on 2 μg of poly$(A)^+$ RNA and either oligo(dT) or internal specific oligonucleotides as primers.

Sequence Analysis

Initial characterization of the DNA and protein sequences was performed with the GCG sequence analysis package (Devereux *et al.*, 1984) and the program DNA Strider (Marck, 1988). The peptide sequence was deduced from DNA sequence by using the ciliate genetic code where UAA and UAG encode glutamine instead of stop (Caron and Meyer, 1985; Preer *et al.*, 1985). The signal sequence and transmembrane helix were identified with the algorithms of von Heijne (1986) and Persson and Argos (1994), respectively. Secondary structure predictions were performed by the methods of Lupas *et al.* (1991), Rost and Sander (1993), Geourjon and Deleage (1993), and White *et al.* (1994). Homology searches were performed with the BLAST program (Altschul *et al.*, 1990).

RESULTS

Cloning of $ND7^+$ by Functional Complementation

The historical difficulty in cloning *Paramecium* genes by functional complementation was mainly due to the nuclear dimorphism of this organism: in the same cytoplasm coexist two diploid germinal micronuclei, transcriptionally silent during vegetative growth, and a highly polyploid (approximately 800 n) somatic macronucleus in which transcription is performed. The macronucleus is degraded at each sexual event and replaced by a new one derived from sublines of the zygotic nucleus. No method is yet available for micronuclear transformation, so it is not possible to obtain permanent transformants. However, the macronucleus can be efficiently transformed by microinjection of purified DNA. It has been shown that *Paramecium* replicates exogenous DNA in its macronucleus whatever its origin, be it linear or circular, cloned into a plasmid or not (Godiska *et al.*, 1987; Bourgain and Katinka, 1991). Taking advantage of this property, Haynes *et al.* (1996) developed a cloning strategy that was based on mutant rescue by total digests of wild-type DNA. Since some 400 genome equivalents can be injected into each macronucleus, any recessive gene is expected to be at least partly rescued. The isolation of the gene is thereafter performed by size-fractionation of the digested DNA, cloning of the rescuing fraction, and sib selection procedure.

We used this strategy herein to clone *ND7*. Wild-type *Paramecium* DNA was digested with each of the following six restriction enzymes, *Bcl*I, *Bgl*II, *Eco*RV, *Hin*dIII, *Swa*I, and *Xba*I, generating fragments of between 1 and 12 kb, and microinjected into nd7–1 mutant cells. We found that *Bcl*I, *Eco*RV, *Hin*dIII, and *Swa*I, but neither *Bgl*II nor *Xba*I, digests were able to restore the exocytotic activity of the mutant. This suggested that only *Bgl*II and *Xba*I have sites within the gene, whereas the others cut outside of the sequence necessary for complementation, as was confirmed later (Figure 3c). Further characterization of the rescuing DNA by size fractionation of the *Bcl*I digest revealed the complementing activity to be in the 4- to 8-kb fraction. After cloning the whole *Bcl*I 4- to 8-kb fraction, the plasmid p201ND7 carrying the gene $ND7^{+}$ in a 5.5-kb insert was isolated by a sib selection procedure involving successive steps of subcloning and tests of mutant rescue by microinjection (Figure 3).

ND7 Gene Sequence

A 1.5-kb open reading frame has been identified by sequencing 3 kb around the central *Bgl*II and *Xba*I sites of the insert of p201ND7 (Figure 4). This sequence is unique in the genome, as deduced from Southern blot experiments. The open reading frame is transcribed since the corresponding cDNA could be amplified by PCR (our unpublished results); the presence of 20- to 33-base small introns characteristic of *Paramecium* (Dupuis, 1992; Russel *et al.*, 1994) was excluded by sequencing the cDNA. The mutation *nd7–1* was sequenced from PCR amplification products of both genomic DNA and cDNA of the mutant. We found the same deletion of a single base, T^{1294}, close to a cluster of thymidines where a thymine dimer could have been induced by the UV irradiation of the mutagenesis. This mutation creates a diagnostic *Ssp*I site, whose presence was confirmed by digestion of a PCR product amplified from mutant DNA (Figure 4).

The Polypeptide ND7p

The polypeptide sequence deduced from the DNA sequence is 506 amino acids long with a predicted molecular mass of 59.3 kDa and an isoelectric point of 5.65. As illustrated in Figure 5, ND7p has a 17-amino acid sequence at its N terminus that could correspond

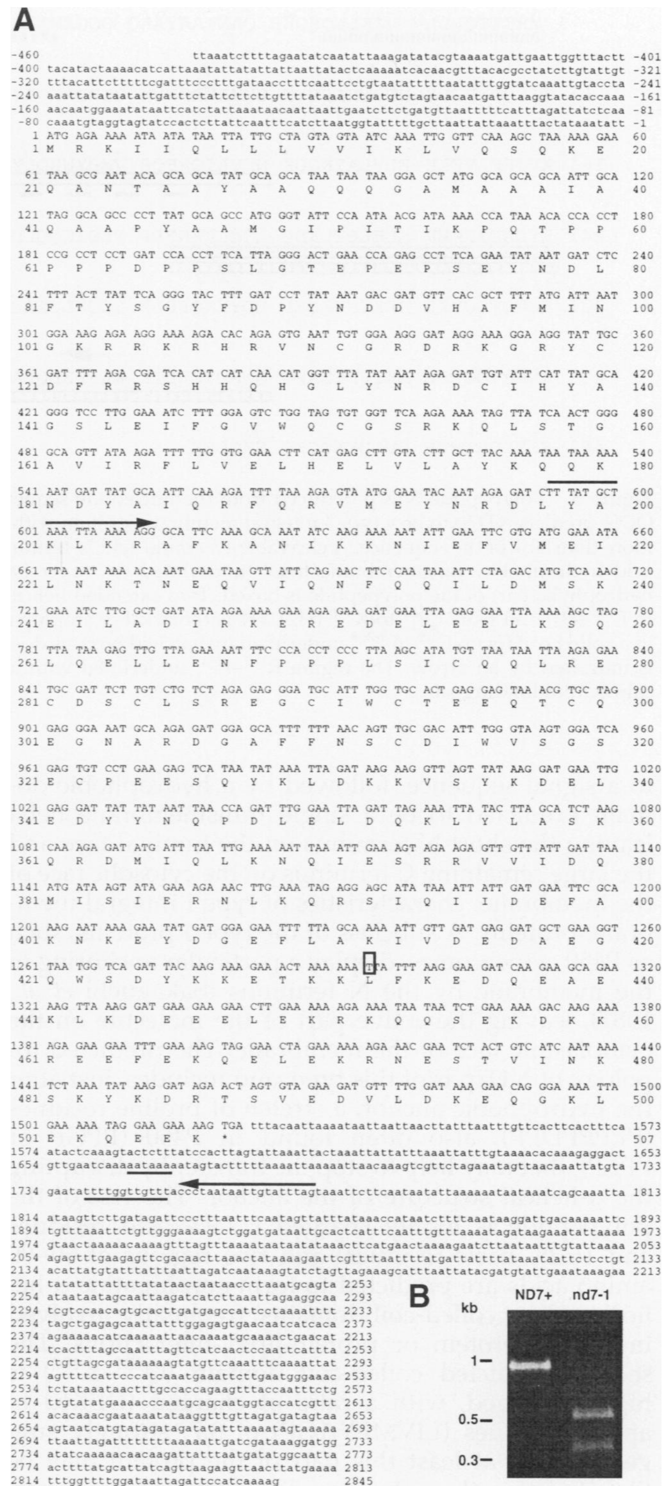

Figure 4. Sequence of the gene *ND7*. (a) The 3-kb of the insert of the p201ND7 was sequenced on both strands by using a series of specific oligonucleotides. A putative polyadenylation site (aataaa followed by a t + g-rich region 60 bp downstream; McDevitt *et al.*, 1986) is underlined. An open reading frame of 506 amino acids was deduced from the DNA sequence with the paramecium genetic code (Caron and Meyer, 1985; Preer *et al.*, 1985). The *nd7–1* mutation

Figure 4 (cont). (deletion of T^{1294}, boxed) creates the *Ssp*I recognition sequence AATATT. (b) Genomic DNA from wild-type and nd7–1 cells were amplified by PCR using oligonucleotides delineated by arrows in a. The amplification products were digested by *Ssp*I and electrophoresed on an agarose gel. The creation of a *Ssp*I site in nd7–1 DNA is apparent.

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  1 MRKIIQLLLV VIKLVQSQKE QANTAAYAAQ QQGAMAAAIA  QAAPYAAMGI PITIKPQTPP PPPDPPSLGT EPEPSEYNDL  80

 81 FTYSGYFDPY NDDVHAFMIN GKRRKRHRVN CGRDRKGRYC  DFRRSHHQHG LYNRDCIHYA GSLEIFGVWQ CGSRKQLSTG 160

161 AVIRFLVELH ELVLAYKQQK NDYAIQRFQR VMEYNRDLYA  KLKRAFKANI KKNIEFVMEI LNKTNEQVIQ NFQQILDMSK 240

241 EILADIRKER EDELEELKSQ LQELLENFPP PLSICQQLRE  CDSCLSREGC IWCTEEQTCQ EGNARDGAFF NSCDIWVSGS 320

321 ECPEESQYKL DKKVSYKDEL EDYYNQPDLE LDQKLYLASK  QRDMIQLKNQ IESRRVVIDQ MISIERNLKQ RSIQIIDEFA 400

401 KNKEYDGEFL AKIVDEDAEG QWSDYKKETK KLFKEDQEAE  KLKDEEELEK RKQQSEKDKK REEFEKQELE KRNESTVINK 480

481 SKYKDRTSVE DVLDKEQGKL EKQEEK
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Figure 5. Polypeptide ND7p deduced from the nucleotide sequence. Computer analysis of the sequence (see MATERIALS AND METHODS) predicts ND7p to be a type I integral membrane protein with a short intraluminal N terminus and a cytosolic C terminus representing more than 80% of the sequence. A cleavable 17-amino acid N-terminal signal sequence (underlined by hatched bar) is followed by a 19-amino acid hydrophobic transmembrane helix (underlined by larger hatching). The proline cluster that interrupts the hydrophobic from the hydrophylic part of the polypeptide is boxed. Two extended helical regions are clearly defined by different prediction algorithms from N^{181} to F^{268} (HI) and from Q^{421} to K^{480} (H2). H1 is remarkably amphipathic (underlined by white box) and has high probability of participating in a coiled coil (from E^{225} to F^{268} underlined by hatched box), as does the H2 helix that contains the *nd7-1* frameshift mutation whose position is indicated by an arrow. The region R^{187}–I^{214}, underlined with a dotted line, is a basic part of the amphipathic α-helix that could be a calmodulin-binding site.

to a signal sequence, followed by a hydrophobic domain predicted to be a single transmembrane helix leaving the short N terminus on the luminal face and the large remaining C terminus on the cytosolic face of the membrane, characteristics of type I integral membrane proteins. A subclass of the type I proteins, such as P450 cytochromes, display a particular anchoring in the membrane by the N terminus (Sakaguchi *et al.*, 1987), leaving the active part of the molecule on the cytoplasmic face of the membrane. The predicted topology of ND7p is of this type, and includes, just after the hydrophobic anchor, a stretch of proline residues (PPPPPDPP), also often found in P450 (PPGP or PPEP), that may help to stop the transfer by disrupting the α-helical structure of the anchor. The rest of the polypeptide is highly charged (17.9% acid residues and 16.2% basic residues) and two regions of 60–90 amino acids are predicted to form amphipathic α-helices and/or coiled-coil domains, frequently involved in protein-protein or protein-lipid interactions. The second predicted coiled-coil domain (Q^{421}–K^{480}) is highly charged, with a ratio of charged (DEKR) to apolar residues (LIVMFYAGP) of 3.7, strongly suggesting that at least this part of the protein may be fibrous rather than globular. The mutation *nd7-1* creates a frameshift moving the first stop codon 300 bp downstream from its normal location. The predicted mutant polypeptide thus has an abnormal C terminus starting after K^{431}.

ND7p was compared with the protein sequence databases (combined nonredundant GenBank/EMBL/SWISS PROT) and to six-phase translations of expressed sequence tags, sequence tagged sites, and the complete yeast genome nucleotide sequence databases, but no homologous sequence was found. *ND7* therefore appears to be a novel gene. It is worth noting that no homologue is found in the genome of yeast, showing that the protein is not present in this species, as expected if the *ND7* gene has evolved to fulfill a specific function in regulated secretion.

DISCUSSION

Paramecium is a unicellular organism that is used as a genetical model for several cell functions absent from most eukaryotic microorganisms, such as membrane excitability, regulated exocytosis, pattern development during morphogenesis, programmed genome rearrangements during differentiation of the somatic nucleus, etc. Numerous mutations affecting each of these functions have been accumulated over the years (Sonneborn, 1974; Görtz, 1988) but only recently has a method for gene cloning by functional complementation been adapted to *Paramecium* by Haynes *et al.* (1996) for a behavioral gene. By using the same technique, we have cloned a gene involved in regulated exocytosis.

The cloning of *ND7* was facilitated by the fact that weak rescue (down to a single discharged trichocyst per cell, Figure 2c), which probably results from microinjection of fewer than 400 copies of the genome during the early steps of the cloning procedure, is

easily detected. Theoretically, the gene corresponding to the mutated allele should be cloned by this method, but it is possible that a different wild-type gene acting as a multicopy suppressor could be isolated during the sib selection steps, as the stoichiometry of the injected DNA molecules with respect to endogenous genes increases. The gene we have cloned is clearly the actual $ND7^+$ gene rather than a multicopy suppressor since 1) the restriction map inside and outside the open reading frame is fully compatible with the results of complementation by total DNA digests (only *Bgl*II and *Xba*I sites are present in the gene, Figure 3) and 2), most importantly, a mutation was found in this gene in the mutant strain nd7–1 (Figure 4).

Consideration of ND7p in light of our previous characterization of the mutant nd7–1 leads us to propose a role for ND7p in exocytotic membrane fusion. 1) Sequence analysis predicts that ND7p is a membrane protein anchored by a single transmembrane helix oriented from the lumen toward the cytosol. It has been shown previously that the site of action of ND7p is the trichocyst compartment (Lefort-Tran *et al.*, 1981). ND7p could thus be an integral membrane protein of the trichocyst with a large cytosolic domain. 2) nd7–1 cells are devoid of connecting material and rosette particles in the plasma membrane (Lefort-Tran *et al.*, 1981) and the assembly of these arrays is known to be induced by the trichocyst while it docks at its site (Beisson *et al.*, 1976; Pape and Plattner, 1985). ND7p is therefore likely to play a role in the assembly of these arrays. 3) The large space (20–30 nm) lying between the two membranes, occupied by the connecting material and the existence of numerous *nd* genes that affect the assembly of this material suggest that ND7p function requires interactions with other proteins. The fact that ND7p is predicted to have amphipathic helices and/or coiled-coil structures in the cytosolic domain argues in favor of such interactions and of ND7p being a key component necessary for assembly of the connecting material and the rosette.

Interestingly, the *nd7–1* mutation is localized in the second putative coiled-coil domain (Figure 5). This may have a particular significance for ND7p function and for the role of the C terminus. However, it is also possible that the frameshift induces global conformational changes preventing the activity of the protein rather than destroying a local structural motif. These two hypotheses can be distinguished in the future by a functional analysis of the sequence by deletion and site-directed mutagenesis.

The study of exocytotic defect in the calmodulin gene mutant *cam1* demonstrated that calmodulin also has a role in the assembly of the connecting material and the rosette (Kerbœuf *et al.*, 1993). The mode of action of calmodulin is always through direct binding and activation of target proteins. No sequence consensus exists for calmodulin-binding motifs but target sequences are in general basic amphipathic α-helices (O'Neil and De Grado, 1990). It is worth noting that the region R^{187}–I^{214} of ND7p (Figure 5, underlined by dotted line) presents the characteristics of a basic amphipathic α-helix. Whether it is an actual calmodulin-binding domain must of course be demonstrated experimentally.

From our database search, we cannot say whether *ND7* homologues exist in higher eukaryotes, as the human and nematode genome sequencing projects are far from complete. That such proteins have not been previously identified in studies of exocytosis is not surprising because they may be very minor components of secretory granules, not easily identified by biochemical methods. Many key factors involved in secretion have been found to be conserved from lower eukaryotes to mammals (Bennett and Scheller, 1993; Burgoyne and Morgan, 1993; Rothman, 1994; Südhof, 1995). It is reasonable to expect that at least some *nd* genes will share sequence homology with known proteins and that the available data from *Paramecium* regulated exocytosis will have general significance.

In metazoa such as the nematode, mutants affecting regulated secretion have also been obtained (Nonet *et al.*, 1993), but since many secreted products are involved in essential processes (i.e., neurotransmission), mutations affecting secretion are expected to be lethal. In *Paramecium*, mutants specifically affected in this process are viable. Thirteen *Paramecium* genes required for exocytosis have already been genetically characterized and the system is far from saturated. Among these, we mention *ND9* coding for a soluble cytosolic factor whose interaction with both trichocyst and plasma membrane has been demonstrated (Beisson *et al.*, 1980) and ND16p (another cytosolic gene product) and ND18p, both of which most likely interact with ND9p, as deduced from the genetic studies (Bonnemain *et al.*, 1992). The cloning of these genes should help our understanding of the nature of the interactions between these proteins and possibly with ND7p and the way in which their predicted coassembly can control membrane fusion. It is clear that regulated exocytosis is a complex process and many of its components remain to be identified; the *Paramecium* system now has the power to allow the discovery of at least some of them.

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