Original

ND7 gene involved in the membrane fusion in regulated exocytosis is conserved in three species of *Paramecium*

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SUMMARY

A *Paramecium tetraurelia ND7* gene homolog was cloned and sequenced in *P. caudatum*. The predicted protein possessed well-conserved regions, a transmembrane helix and two coiled-coil domains possibly involved in protein-protein interaction, as previously suggested for *P. tetraurelia.* Southern hybridization revealed that the *ND7* gene homolog was also present in *P. multimicronucleatum,* suggesting that the ND7p is well-conserved and plays an important role in regulated exocytosis in *Paramecium* species. We treated cells with lysozyme to discharge most of the trichocysts and incubated cells to regenerate them. The ND7 mRNA was present only in a trace amount in cells with abundant trichocysts, while, it drastically increased in trichocyst-regenerating cells. This work also provides a useful system to isolate genes/proteins which are involved in trichocyst discharge in *Paramecium*.

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INTRODUCTION

Regulated exocytosis is a particular phenomenon in which secretory products are released in response to specific extracellular stimuli, through a complex mechanism involving a large number of proteins. This process is restricted to specialized cell types, such as neuronal and endocrine cells in metazoa, and the mechanism has not been well elucidated. The ciliate *Paramecium*, a unicellular organism, undergoes the well-known regulated exocytosis. The secretory granules called trichocysts are synthesized in the cytoplasm, transported to the cell surface, and embedded at the specific docking site in the plasma membrane. The specific stimuli, such as attack of the predators (Harumoto & Miyake, 1992), chemicals e.g. saturated picric acid (Adoutte, 1988, for review), aminoethyl dextran (Plattner et al. 1985) and lysozyme (Harumoto & Miyake, 1992), and certain physical or electrical stimuli, trigger the fusion of the plasma membrane and the trichocyst membrane, and finally the contents are exocytosed to outside of the cell. The mechanism of this regulated exocytosis in *Paramecium* has been well investigated morphologically and genetically. The specialized docking sites are characterized by intramembranous particles or 'rossette', in the plasma membrane just above the

trichocyst tip, and the fibrous structure, 'connecting material' which links the plasma membrane and the trichocyst membrane (Plattner et al., 1973; Beisson et al., 1976). In *P. tetraurelia*, 23 recessive mutants (nd: non-discharge) have been isolated and the mutations mapped 13 different loci. These mutants affect the final step of exocytosis, including membrane fusion of the plasma membrane and the trichocyst membrane. The genetic analysis revealed that the rosette and the connecting materials were involved in the membrane fusion. Microinjection experiments also revealed the site of action of the products of the *ND* genes; *ND7* and *ND9* gene products were localized in the trichocysts and the cytosol, respectively. The trichocysts of the nd7 mutant are normally docked under the plasma membrane, but are not able to discharge. Imperfect arrangement of the rosette and the connecting materials were associated in this mutation.

Recently the *ND7* gene in *P. tetraurelia* was isolated by functional complementation, and sequenced (Skouri & Cohen, 1997). The *ND7* gene product is 506 amino acids long with a predicted molecular mass of 59.3 kDa consisting of 16-amino acid sequences of a signal peptide in N-terminus, followed by a short hydrophobic domain and remaining large portion of hydrophilic region, which is characteristic to the type I integral membrane proteins. The nd7 mutation is the deletion of T^{1294} which creates a frameshift resulted in abnormal C terminus after K^{431}. No homologous gene has been found in the database, suggesting that ND7p is a novel protein specific to the regulated exocytosis. To elucidate the ND7p function, it would be interesting to see whether *ND7* gene is conserved in other species of *Paramecium*.

In this study, we represent that two other species of *Paramecium* possess the *ND7* gene homologs, and show the complete sequence of *ND7* homolog in *Paramecium caudatum*, and then we discuss the possible function of the *ND7* gene product. This study also represents a useful method to isolate genes/proteins involved in trichocyst discharge.

MATERIALS AND METHODS

Stocks

Stocks 51, nd7, nd9 of *P. tetraurelia*, stocks NT1, 27aG3, KNZ-5 of *P. caudatum* and stock 49B of *P. multimicronucleatum* were used. Stocks 51, NT1, KNZ-5, 49B were wild-type, and nd7, nd9 and 27aG3 were trichocyst non-discharge mutants. All these mutants possess trichocysts but are incapable of discharging them.

Culture conditions

Cells were grown at 25°C in wheat grass powder (Pines, Lawrence, KS) infusion, bacterized two days before use with *Enterobacter aerogenes* (Takagi, et al. 1996).

Purification of genomic DNA

Cells in the early stationary phase were collected in the pear-shaped centrifuge tube and incubated with 2 volumes of low-EDTA lysing solution (10 mM Tris-HCl, 1% SDS, 50 mM EDTA, pH 9.5) (Harumoto, 1986) at 65°C for 20 min. Dissolved cells were treated with phenol, and purified DNA was treated with RNase and Proteinase K.

Extraction of RNA and cDNA synthesis

Total RNA was obtained from 1×10^5 cells in the early stationary phase. Cells were treated by Trizol reagent (Gibco life technologies) and mixed with chloroform, and RNA was extracted. $PolyA^+$ RNA was isolated using oligotex-dT30 (super) kit (Roche Molecular Biochemicals), and cDNA was synthesized using SMART RACE cDNA amplification kit (Clontech).

Plasmids

Plasmid pGemND7(+) was kindly provided by

Table 1 Nucleotide sequences of primers used in this study

primer	nucleotide sequences
ND7_043F	5'-TAGGATCCGCNCCNTAYGCNGCNATG-3'
ND7_093F	5'-TAGGATCCGAYGTNCAYGCNTTYATG-3'
ND7_144F	5'-TAGGATCCGARATHTTYGGNGTNTGG-3'
ND7_187F	5'-TAGGATCCMGNTTYYARMGNGTNATG-3'
ND7_266F	5'-TAGGATCCGARAAYTTYCCNCCICC-3'
ND7_448R	5'-TAGGATCCYTCYTRRTCYTCYTTRAA-3'
ND7_506R	5'-TAGGATCCYTTYTCYTCYTRYTTYTC-3'
ND7_F1	5'-GCTGATGAAGATGCTGAGGGCAAATGGGGTG-3'
ND7_F2	5'-GAAGACCAAGATGAGAGAAAAGAAAAGGATGC-3'
ND7_R1	5'-GGCTCCATCCCTCGCAGTTCCCTCC-3'
ND7_R2	5'-CCTGTGTACACCAAACACATCCTTCTCTTG-3'
P.c_ACT153F	5'-AGCTTACGTTGGTGATGAGG-3'
P.c_ACT821R	5'-AGACCTGACACTTCAAGACC-3'

Primers ND7_043F, ND7_093F, ND7_144F, ND7_187F, ND7_266F, ND7_448R, and ND7_506R are degenerate primers designed from *ND7* gene of *P. tetraurelia*. ND7_F1, ND7_F2, ND7_R1, ND7_R2 are identical primers designed from the sequenced 700-bp fragment (see Result section). P.c_ACT153F and P.c_ACT821R are identical primers designed from actin gene of *P. caudatum* (AB070223).

Dr. Jean Cohen. The plasmid (5 kb) contains 1.5-kb open reading frame of *ND7* gene (Skouri & Cohen, 1997). The *ND7* gene with 5'- and 3' flanking region is integrated in pGemND7(+) as a 2-kb *Sph* I - *Pst* I fragment.

Southern hybridization

Genomic DNAs of *P. tetraurelia*, *P. caudatum* and *P. multimicronucleatum* were digested by *Bcl*I, electrophoresed, blotted and hybridized with DIG-labeled pGemND7(+) DNA. The probe was synthesized by DIG DNA labeling kit (Roche Molecular Biochemicals).

Dot hybridization

Five micrograms of total RNA was placed on the nylon membrane, dried and treated by 50 mM NaOH. The membrane was irradiated by UV for 10 min and hybridized with DIG-labeled 2 kb *Sph* I - *Pst* I fragment of pGemND7(+).

PCR, cloning products, and sequencing

Table 1 shows the primers which we used in this study. Degenerate primers were designed from the amino acid sequence of *ND7* gene of *P. tetraurelia* (Skauri & Cohen, 1997). The identical primers were designed from the sequenced 700-bp fragment described in Result section. The PCR amplification was carried out using Advantage 2 Polymerase Mix and PCR kit (Clontech) on a Perkin-Elmer 9700 thermal cycler with a start at 95°C for 1 min followed by 35 cycles at 95°C (30 sec), 49°C (30 sec) and 72°C (1 min). PCR products were electrophoresed, purified from the gel and cloned in pCR2.1-TOPO vector (Invitrogen). Sequence reaction was performed using ABI Prism Big Dye terminator cycle sequencing ready reaction kit.

Induction of trichocyst regeneration

Cells suspended in SMB- (Sugiura & Harumoto, 2001) were mixed with lysozyme (final conc.

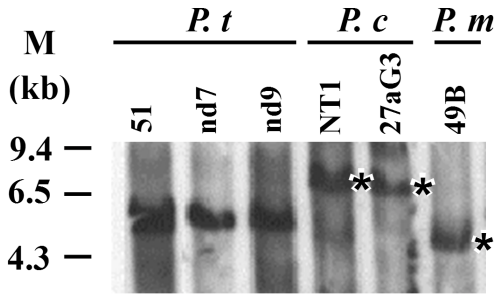

Fig. 1. Detection of *ND7* homologs in *P. caudatum* and *P. multimicronucleatum*. *P.t*: *P. tetraurelia*, stocks 51, nd7 and nd9. *P.c*: *P. caudatum*, stocks NT1 and 27aG3. *P.m*: *P. multimicronucleatum*, stock 49B. Asterisks show the expected bands as *ND7* gene homologs in *P. caudatum* and *P. multimicronucleatum*. The filter was hybridized at 50°C.

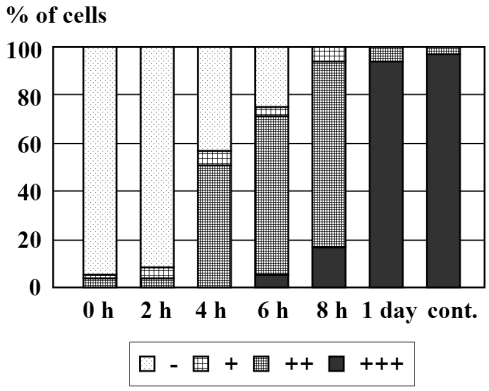

Fig. 2. Regeneration of trichocysts. Cells (*P. caudatum,* stock KNZ-5) treated with 100 µg/ml of lysozyme for 30 sec were incubated in SMB-. The regenerated trichocysts were discharged by treating cells with saturated picric acid. The extent of trichocyst discharge was classified into four categories (Harumoto & Miyake, 1992): – (no discharge), + (discharge of up to three trichocysts), ++ (intermediate discharge), +++ (full discharge). In the full discharge (+++), a fixed cell is fully surrounded by discharged trichocysts. (++) is between + and +++. One hundred cells were counted in each sample. Cells were examined at 0, 2, 4, 6, 8 hours, and 1 day after the treatment with lysozyme. cont.: cells without treatment.

100 µg/ml) solution and incubated for 30 sec at the room temperature. Lysozyme induces trichocyst discharge without killing cells (Harumoto & Miyake, 1992). The cells were washed and the discharged trichocysts were removed by centrifugation with excess amount of SMB-. Cells were then resuspended in fresh SMB- and incubated at 25°C for trichocyst regeneration.

Monitoring of exocytosis

The competence of exocytosis was evaluated by the extent of discharging trichocysts. Cells were treated with saturated picric acid, and the discharged trichocysts were observed under the stereomicroscope. Saturated picric acid is not only a fixative but a strong secretagogue to induce most of the trichocysts in the cell. The extent of trichocyst discharge was classified into four categories (Harumoto & Miyake, 1992): – (no discharge), + (discharge of up to three trichocysts), ++ (intermediate discharge), +++ (full discharge). In the full discharge (+++), a fixed cell is fully surrounded by discharged trichocysts. (++) is between + and +++. One hundred cells were counted in each sample.

RESULTS

Identification of *ND7* homolog in *P. caudatum* and *P. multimicronucleatum* by Southern hybridization

We detected the expected band of *ND7* gene about the size of 5.5 kb (Skauri &Cohen, 1997) in stocks 51, nd7 and nd9 in *P. tetraurelia* in the stringent condition (68°C). In *P. caudatum* and *P. multimicronucleatum*, the corresponding band was not detected when hybridized at 68°C and 55°C, but it appeared at 50°C. The band was about the size of 6.5 kb in *P. caudatum* (Stocks NT1 and 27aG3) and 4.5 kb in *P. multimicronucleatum* (stock 49B) (Fig. 1). The results suggested that *P. caudatum* and *P. multimicronucleatum* possessed the *ND7* gene

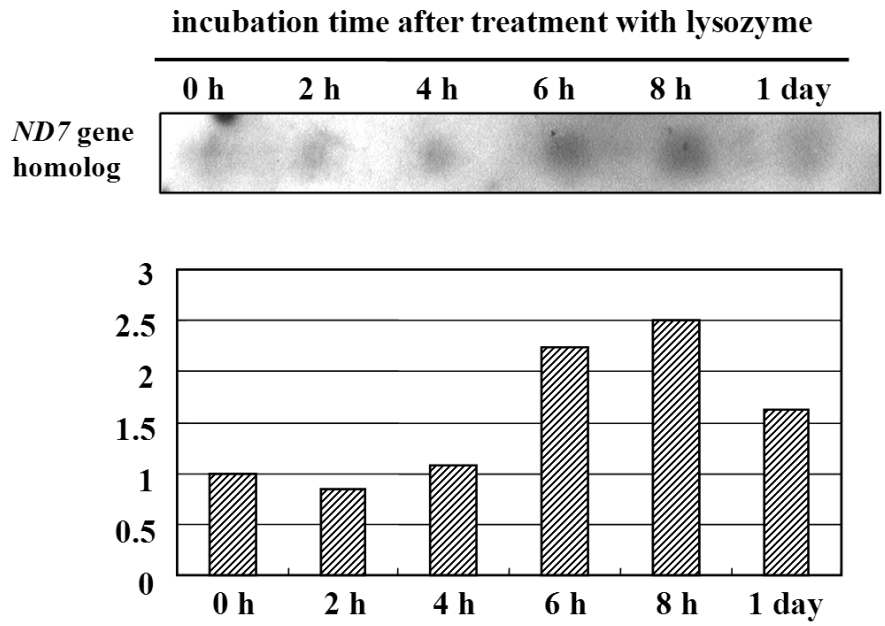

Fig. 3. Dot hybridization of *ND7* homolog mRNA. Total RNA (5μg/ml) extracted from cells pretreated with lysozyme and incubated in SMB- for 0, 2, 4, 6, 8 hours and 1 day, was dotted in a filter and hybridized with the DIG-labeled *ND7* gene prove. Graph represents the relative amount of *ND7* homolog mRNA as the amount of mRNA in 0h is 1.

homologs.

Regeneration of trichocysts and expression of *ND7* gene homolog

Preliminary experiments using cDNA as templates, prepared from cells of *P. caudatum* in the early stationary phase, with possible combinations of degenerate primers failed to amplify any band by PCR. The result might be due to insufficient amount of mRNA of the *ND7* gene homolog in the cells. To obtain abundant mRNA of the *ND7* gene homolog, we attempted to increase the amount by inducing trichocyst regeneration. We artificially removed trichocysts from cells by treating them with 100 μg/ml lysozyme for 30 sec. Fig. 2 shows the time course of trichocyst regeneration in the treated cells. Immediately after the treatment, most of the cells lost the capacity to discharge trichocysts, and the capacity gradually recovered with time. After 4, 6, 8 hours after the treatment, about 50%, 70% and 90% of cells possessed relatively large amount of trichocysts (++ and +++), respectively. We extracted RNA from these cells and subjected them for the dot hybridization (Fig. 3). The dot was only faint in 0 and 2 hours, which gradually became distinct in 4-8 hours, and faded again in 1 day. It suggests that the mRNA of the *ND7* gene homolog is present only in a trace amount in both untreated cells (cont.) and cells incubated in SMB- for up to 2 hours after the treatment with lysozyme, and it gradually increases with the time elapses and reaches the peak at about 6-8 hours.

Cloning and sequencing of *ND7* homolog

Total RNA was extracted from the lysozyme-treated cells after 8-hours incubation in SMB-. PCR reaction was performed using the synthesized cDNA as a template with several possible combinations of degenerate primers (Table 1). PCR

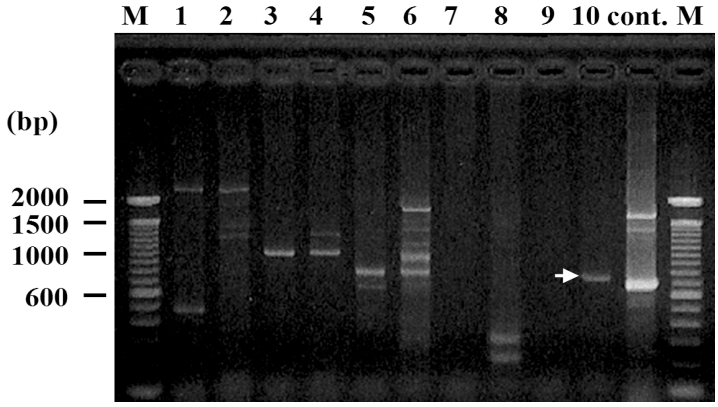

Fig. 4. Amplification of *ND7* gene homolog in *P. caudatum*. Arrow (lane 10) indicates the expected band about the size of 700 bp. As a positive control (lane cont.), actin gene primers were used. The primers used in each lane are: (lane 1) ND7_043F, ND7_448R, (lane 2) ND7_043F, ND7_506R, (lane 3) ND7_093F, ND7_448R, (lane 4) ND7_093F, ND7_506R, (lane 5) ND7_144F, ND7_448R, (lane 6) ND7_144F, ND7_506R, (lane 7) ND7_187F, ND7_448R, (lane 8) ND7_187F, ND7_506R, (lane 9) ND7_266F, ND7_448R, (lane 10) ND7_266F, ND7_506R, (lane cont.) P.c_ACT153F and P.c_ACT821R, M: DNA size marker.

products were identified in several combinations of primers (Fig.4, lanes 1,2,3,4,5, 6,8,10). We cloned and sequenced a single clearly-identified band (lane 10) about the size of expected 700 bp. The sequence of this band had homology to *ND7* gene of *P. tetraurelia*. The identical primers were designed from the sequence of this 700 bp-fragment, and finally 1521 bp of whole *ND7* gene homolog was sequenced.

Common properties of *ND7* gene

The sequenced *ND7* gene homolog of *P. caudatum* had 74.1% homology in nucleotides and 75.9% homology in amino acids to *ND7* gene of *P. tetraurelia* (Fig.5). The predicted amino acid sequence of the *ND7* gene homolog consists of 506 amino acids same as *ND7* gene in *P. tetraurelia* with a molecular mass of 59.1 kDa and an isoelectric point of 5.38. As shown in Fig.5, ND7p has a 16-amino acid sequence at N terminus which corresponds to a signal sequence, followed by a hydrophobic region predicted to be a single transmembrane helix. The result supports the previous suggestion that the ND7p is a type I integral membrane protein, leaving the short N terminus on the luminal face and the large remaining C terminus on the cytosolic face. The stretch of proline residues (PPPDPEPP) which is required for disruption of the α-helical structure of the anchor in the type I integral membrane protein, was also found. The rest of the polypeptide is highly charged (20.2% acid residues and 19.1% basic residues) and two regions of amphipathic α-helices and/or coiled-coil domains, possibly involved in protein-protein or protein-lipid interactions are well conserved.

DISCUSSION

This study revealed that the *ND7* gene homolog is present in *P. caudatum*, and possibly in *P. multimicronucleatum*. We cloned and sequenced the *ND7* gene homolog in *P. caudatum* and showed that the amino acid sequence was well conserved in *Paramecium* two species suggesting the important role of ND7p in trichocyst discharge.

Skouri and Cohen (1997) suggested the possible role of ND7p; 1) ND7p is a membrane protein, anchored by a single transmembrane helix, 2) ND7p is possibly involved in assembly of connect-

Fig. 5. Deduced amino acid sequence of *ND7* gene homolog of *P. caudatum* (*P. c*) aligned with *P. tetraurelia* (*P. t*). Characteristic regions were represented in the boxes. Open box: signal peptides, box with broken line: hydrophobic transmembrane helix, box with dotted line: proline cluster, double-lined boxes: amphipathic helices and/or coiled-coil domains.

ing materials and rosette particles, 3) ND7p is likely to interact with other proteins. The results obtained in this study also support the hypothesis, because the sequence of ND7 homolog represented single transmembrane helix, and regions of amphipathic α -helices and coiled-coil domains. It is interesting to delete or mutate well-conserved sequences to identify the region which is indispensable and necessary for the protein function.

This work also represents a useful method to isolate genes involved in trichocyst discharge. Trichocysts, once removed from the cell, regenerate within several hours. We treated cells with lysozyme as a secretagogue to induce trichocyst discharge, incubated them for regeneration, and then obtained sufficient amount of mRNA. The results

indicated that only a small amount of the *ND7* gene was transcribed in untreated cells, and the mRNA became abundant during trichocyst regeneration at 4-8 hours after the treatment.

Trichocysts, the exocytotic organelles of *Paramecium* provide a useful system to isolate genes/proteins involved in the exocytosis. The additional information about *ND7* gene and the method represented in this study will be advantageous for further investigation.

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