Mechanisms of disease

Rescue of defective pancreatic secretion in cystic-fibrosis cells by suppression of a novel isoform of phospholipase C

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Summary

Background Cystic fibrosis is caused by mutations in the gene encoding an ion-transport protein, the cystic-fibrosis transmembrane conductance regulator (CFTR). Defective secretion of anions is the primary cause of many of the clinical manifestations of cystic fibrosis, including pancreatic insufficiency. We aimed to identify a molecular mechanism from which a new method to circumvent defective pancreatic secretion could be derived.

Methods Multiple-human-tissue RT-PCR and semiquantitative RT-PCR analyses were used to examine gene expression. An antisense technique was used in conjunction with radioimmunoassay, Fura-2 spectrofluorometry, immunohistochemistry, and the short-circuit current technique (Ussing chamber) for elucidation of gene function and its application in rescuing defective pancreatic secretion.

Findings We cloned a newly identified gene, NYD-SP27, which has structural similarity to an isoform of phospholipase C. NYD-SP27 was expressed endogenously in human pancreatic-duct cells and upregulated in cystic fibrosis. Suppression of NYD-SP27, by transfection of its antisense into human cystic-fibrosis pancreatic-duct cells, resulted in augmentation of phospholipase-C-coupled calcium-ion release and protein kinase C activity, improvement in the amount of mutated CFTR reaching the plasma membrane, and restoration of cAMP-activated pancreatic anion secretion.

Interpretation NYD-SP27 exerts an inhibitory effect on phospholipase-C-coupled processes that depend on calcium ions and protein kinase C, including CFTR trafficking and function. Its upregulation in pancreatic-duct cells may reveal a previously unsuspected defect in cystic fibrosis contributing to pancreatic insufficiency, and thus represents a new target for pharmacological intervention in cystic fibrosis.

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Introduction

Cystic fibrosis is a common genetic disease among the white population. It is characterised by defective secretion of chloride and bicarbonate ions in most exocrine glands and organs, especially the lungs, caused by mutations in the gene encoding the cystic-fibrosis transmembrane conductance regulator (CFTR). Nearly 90% of patients with cystic fibrosis have pancreatic insufficiency. CFTR is a low-conductance chloride channel activated by phosphorylation of the regulatory domain by protein kinases A and C, and gated by ATP hydrolysis at two nucleotide-binding domains. The most common cystic-fibrosis-associated mutation (60–70% of cases) is a deletion of the phenylalanine residue at position 508 (ΔF508) in nucleotide-binding domain 1. This mutation causes protein-trafficking defects, and most of the transporter fails to progress to the Golgi apparatus and cell membrane. This mutation also has an adverse functional effect; although a small proportion of the channel reaches the cell membrane, the probability that the channel will open in response to CAMP stimulation is lower than that for wild-type channels. Most research efforts have focused on pharmacological methods to rectify the defective CFTR trafficking and function.

In this study, we investigated a newly identified gene, NYD-SP27. The gene is expressed endogenously in human pancreas and upregulated in cystic fibrosis, which suggests that it is involved in the pathogenesis of the disorder. NYD-SP27 has structural characteristics similar to those of the phospholipase C group of proteins. These enzymes cleave the polar head group from inositol phospholipids giving rise to two intracellular messengers, diacylglycerol and inositol 1,4,5-triphosphate, which activate protein kinase C and cause calcium-ion mobilisation, respectively.

Methods

Experimental procedures Normal human tissue distribution of NYD-SP27 was investigated by PCR of human MTC Panel I and II kits (Clontech, Palo Alto, CA, USA). Semiquantitative RT-PCR was done with an annealing temperature of 55°C. The primers for human and murine tissue are given in the panel. β actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as controls for human being and mouse, respectively.

The full-length cDNA of NYD-SP27 was subcloned into pCI-neo (Promega, Madison, WI, USA) to construct the sense vector. The antisense vector was generated by inserting a fragment (370–615 bp, with no homology to other known isoforms of phospholipase C) of NYD-SP27 into pCI-neo vector in reverse orientation. Pancreatic-duct cells from patients with cystic fibrosis (2×10^6 per well) were seeded into six-well plates and transfected by use of lipofectin reagent (Gibco, Carlsbad, CA, USA) according to the manufacturer’s instructions with sense
and antisense vectors. Iscove’s modified Dulbecco’s medium containing G418 (400 μg/mL) was used to select the stable transfected clones, which were further confirmed by semi-quantitative RT-PCR.

Intracellular concentrations of free calcium ions were measured with Fura2/AM. The fluorescent signal was monitored by an LS-50B luminescent spectrometer (Perkin Elmer, Boston, MA, USA). The excitation light at 340 nm and 380 nm was provided by a 150 W xenon arc lamp and a filter wheel containing 340 nm and 380 nm interference filters. The emitted fluorescence at 510 nm was collected by a photomultiplier tube and recorded. The cells were loaded with 5 μmol/L Fura2 by incubation for 45 min in darkness at 37°C in phosphate-buffered saline containing 2 mmol/L calcium chloride, pH 7·4. The cells were washed and resuspended and incubation for 45 min in darkness at 37°C in phosphate-buffered saline, the cells were incubated with 2 mmol/L calcium chloride, pH 7·4. The cells were washed and resuspended in phosphate-buffered saline. After three washes with phosphate-buffered saline, the cells were incubated with secondary antibody to murine IgG conjugated to fluorescein isothiocyanate (1 in 200 dilution) with 1% normal goat serum in phosphate-buffered saline. After 15 min in darkness at 37°C, the cells were then washed and resuspended in phosphate-buffered saline with or without 2 mmol/L calcium chloride before the experiments.

The measurement of SHORT-CIRCUIT CURRENT (Isc) has been described previously.\(^1\) Monolayers grown on permeable supports were clamped vertically between the two halves of an Ussing chamber. The monolayers were bathed with Krebs-Henselet solution, bubbled with 95% oxygen and 5% carbon dioxide to maintain the pH of the solution at 7·4 and maintained at 37°C. Isc was measured with silver/silver chloride reference electrodes (World Precision Instruction, Sarasota, FL, USA) connected to a preamplifier and a voltage-clamp amplifier (DVC-1000). Precision Instruction, Sarasota, FL, USA) connected to a Zeiss Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany) with the blue filter set. The digital images were stored on Jaz disks (Iomega, Roy, UT, USA) for subsequent analyses.

**Statistics**

Comparisons between groups of data were made either by Student’s t test (two-group comparison) or one-way ANOVA with Newman-Keuls post-hoc test (three-group comparison).

**Role of the funding source**

The funding sources had no role in the study or the decision to submit the report for publication.

**Results**

We identified from a human adult testis cDNA library by differential hybridisation technology a novel gene, NYD-SP27 (Genbank accession number AY035866), of 2132 bp, which encodes a protein of 504 amino acid residues.\(^2\) A blast search with the aminoacidic sequence of NYD-SP27 showed striking structural similarity to the known and putative forms of phospholipase Cδ from human beings, *Macaca fascicularis*, *Mus musculus*, *Rattus norvegicus*, and cattle. As with other phospholipases Cδ, NYD-SP27 has three conserved domains, Xc, Yc, and C2 (order of domains: amino terminus, Xc, Yc, carboxyl terminus).\(^3\) NYD-SP27 has a short carboxyl terminus, as do other phospholipases Cδ. Unlike the others, however, it does not have the PLECKSTRIN HOMOLOGY DOMAIN or EF hand motifs in its amino terminus.

RT-PCR showed strong expression of NYD-SP27 in the testis and a weak band in the pancreas, but the gene was not expressed in any of the other 14 tissues examined (figure 1A). Semi-quantitative RT-PCR analysis showed upregulated expression of NYD-SP27 in pancreatic-duct cells from patients with cystic fibrosis (CPAC-1) and pancreas of cystic-fibrosis model mice (CFTR\textsuperscript{min/+}; figure 1B) compared with their normal counterparts, suggesting that it could be involved in the pathogenesis of cystic fibrosis.
An antisense vector was constructed and stably transfected into pancreatic-duct cells from patients with cystic fibrosis (AT-CF) to interrupt the expression of NYD-SP27 (confirmed by semiquantitative RT-PCR; data not shown). Extracellular ATP is known to activate purinergic receptors coupled to various isoforms of phospholipase C, leading to both activation of protein kinase C and mobilisation of calcium ions. Therefore, involvement of NYD-SP27 in the phospholipase C signalling pathway could be shown by measurement of ATP-induced mobilisation of calcium ions and stimulation of protein kinase C activity in NYD-SP27 antisense-transfected cystic-fibrosis cells (AT-CF). AT-CF cells had a significantly higher mean basal fluorescence ratio (2·09, SE 0·03), indicating the intracellular calcium-ion concentration, than the control-vector-transfected cystic-fibrosis (VT-CF) cells (1·92, 0·03) and sense-transfected NYD-SP27-overexpressed cystic-fibrosis (ST-CF) cells (1·83, 0·02; figure 1C). Furthermore, extracellular ATP (100 µmol/L) in the absence of extracellular calcium ions induced a significantly greater increase in intracellular calcium concentration in AT-CF cells (47·6%, 6·5) than in VT-CF (19·4%, 4·4) or ST-CF cells (17·6%, 2·3; figure 1D) and a significantly larger increase in activity of protein kinase C (11·4 [1·4] times vs 2·0 [0·8] and 0·7 [0·10] for VT-CF and ST-CF; figure 1E). Thus,
phospholipase-C-coupled calcium mobilisation and protein kinase C activity were suppressed by NYD-SP27 overexpression, but increased by suppression of the gene. Ussing chamber experiments measuring transepithelial currents (short-circuit current, $I_{SC}$) to examine the effect of NYD-SP27 on pancreatic anion secretion showed that apical application of ATP (10–100 μmol/L) stimulated an $I_{SC}$ response with fast transient kinetics in VT-CF or ST-CF cells (figure 2A), which was similar to the previously observed ATP-induced and calcium-dependent anion-mediated $I_{SC}$ in non-transfected cells.14,15 However, a more sustained rise with slower decline was observed in AT-CF cells, which resembled the cAMP-dependent anion secretion induced by either forskolin or secretin previously observed in normal pancreatic-duct cells.16 The averaged total charge transfer (area under the curve) across AT-CF cells was three times larger than that in the control VT-CF ($p<0.001$), whereas the ATP-induced response in NYD-SP27-overexpressed ST-CF cells was significantly smaller than that of VT-CF ($p<0.001$), indicating an inhibitory role of NYD-SP27 in phospholipase-C-coupled anion secretion.

The increased ATP-induced $I_{SC}$ in AT-CF cells and the sustained nature of the $I_{SC}$ suggested possible involvement of cAMP. Addition of MDL12330A (20 μmol/L), an inhibitor of adenylate cyclase, led to 81% inhibition of the ATP-induced current in AT-CF ($p<0.0001$) but not VT-CF cells (figure 2B), indicating that a substantial proportion of the ATP-induced current in NYD-SP27-suppressed AT-CF cells was mediated by cAMP.

We then examined whether a cAMP-activated $I_{SC}$ response could be induced directly in AT-CF cells with forskolin (10–50 μmol/L), an activator of adenylate cyclase. There was a large increase (4702 μC/cm², 765) in $I_{SC}$ with continuously rising kinetics in AT-CF cells, which had not been previously observed in normal pancreatic-duct cells; however, addition of forskolin resulted in far less prominent increases in VT-CF (1257 μC/cm², 225) and ST-CF (284 μC/cm², 92) cells (figure 3). The forskolin-induced $I_{SC}$ response was greatly potentiated by previous stimulation with ATP in AT-CF (figure 4A) but not VT-CF or ST-CF cells (not shown). In the presence of U73122, an inhibitor of phospholipase C, or staurosporine, an inhibitor of protein kinase C, the ATP-potentiated forskolin-induced $I_{SC}$ was significantly lower ($p=0.0005$ and $p=0.0003$, respectively), which shows the involvement of phospholipase C and protein kinase C in the potentiation of the forskolin response by ATP. The ATP-potentiated forskolin response was also blocked by the specific

![Figure 2: ATP- induced $I_{SC}$ responses](image)

**Figure 2:** ATP- induced $I_{SC}$ responses

A: ATP stimulated $I_{SC}$ change in VT-CF, ST-CF, and AT-CF. B: summary of ATP-induced $I_{SC}$ responses, expressed as changes in total charges transferred over 30 min, showing inhibition by adenylate-cyclase inhibitor MDL12330A in AT-CF but not VT-CF, $p$ value for comparison of ATP alone vs ATP and MDL12330A in AT-CF cells.

![Figure 3: Forskolin-induced $I_{SC}$ responses](image)

**Figure 3:** Forskolin-induced $I_{SC}$ responses

A: representative forskolin-induced $I_{SC}$ recordings in AT-CF and VT-CF cells. B: summary of forskolin-induced $I_{SC}$ response. $p$ values are for comparison with AT-CF.
adenylate-cyclase inhibitor Rp-cAMP (not shown) and diphenylamine 2,2'-dicarboxylic acid or glibenclamide, both of which are known to block CFTR, but not by 4,4'-diisocyanatostilbene 2, 2' disulphonic acid (figure 4B), which blocks various chloride-ion channels including calcium-activated channels but not CFTR. Thus, the forskolin-induced $I_{Na}$ seemed to be mediated by CFTR, indicating possible rectification of defective CFTR by suppression of NYD-SP27.

$\Delta F508$-CFTR protein, which is retained in the endoplasmic reticulum owing to misfolding, can function as a cAMP-dependent chloride-ion channel if it is able to reach the cell membrane (eg, by changes in the calcium-ion concentrations of its intracellular pool). The rescuing effect on defective pancreatic anion secretion by suppression of NYD-SP27 could therefore be due to rectification of CFTR trafficking. To test this hypothesis, we examined surface expression of $\Delta F508$-CFTR in AT-CF, VT-CF, and ST-CF cells by immunolocalisation. CFTR immunostaining was found predominantly in the cytoplasm in VT-CF and ST-CF cells (figure 5), indicating retention of CFTR in the endoplasmic reticulum. However, in AT-CF cells, CFTR immunostaining showed a ring-like pattern around the cells, indicating the presence of CFTR in the plasma membrane when NYD-SP27 was suppressed.

**Discussion**

The majority of patients with cystic fibrosis, if untreated, suffer severe malnutrition caused mostly by pancreatic insufficiency, which is thought to be a consequence of a primary defect in electrolyte and fluid secretion. The volume and bicarbonate content of the pancreatic secretion are much lower than normal in cystic fibrosis, and most patients are refractory to stimulation with secretin, a cAMP-evoking hormone that is the principal agent bringing about upregulation of pancreatic bicarbonate secretion. Protein precipitation and obstruction within small pancreatic ducts, due to the lack of pancreatic fluid resulting from defective secretion of chloride and bicarbonate ions, are thought to produce the pancreatic acinar atrophy and fibrosis seen in the disorder. This process is one of the major causes of the development of pancreatic insufficiency that accounts for the steatorrhoea observed in over 85% of people with cystic fibrosis.

The discovery of NYD-SP27, its inhibitory effect on phospholipase-C-coupled mobilisation of calcium ions and activity of protein kinase C, and the apparent rectifying effect of NYS-SP27 antisense on CFTR trafficking and function have revealed a previously unsuspected molecular mechanism that may also contribute to the pathogenesis of pancreatic insufficiency in cystic fibrosis. Phospholipase C signal transduction is known to have many cascades of signalling pathways involving different isoforms of the enzyme. Phospholipase Cβ is generally thought to be the first isoform involved in the initial activation by G proteins coupled to various receptors, including purinergic receptors for extracellular ATP. Different isoforms of phospholipase C, including phospholipase Cβ, are thought to be involved, either potentiating or activating one or more of the subsequent cascades of signalling pathways leading to activation of either or both calcium mobilisation and protein kinase C. The structural difference between NYD-SP27 and other previously...
identified phospholipases C suggests a possible difference in mode of action. The suppression of calcium mobilisation and protein kinase C activity in NYD-SP27-overexpressed cells (ST-CF) and the rise in these two activities in antisense-transfected cells suggest that NYD-SP27 acts as a negative modulator of the phospholipase C signalling pathways, in contrast to the potentiating role found for SP27 previously identified in CHO cells.

Both calcium ions and protein kinase C are known to be involved in a range of important pancreatic functions, including secretion of digestive enzymes and anions. NYD-SP27, as a negative modulator, could therefore provide a means to fine-tune phospholipase-C-coupled physiological responses on stimulation, such as by extracellular ATP, a physiological regulator of the pancreas known to activate both phospholipase-C-coupled calcium-ion and protein-kinase-C dependent pathways.14,15 Expression of NYD-SP27 in the pancreas is normally very low (figure 1A), suggesting a minor role for the inhibitory NYD-SP27 in normal pancreatic function.

The upregulated NYD-SP27 expression found in the pancreas in cystic fibrosis suggests that the protein may interfere with pancreatic function, adding to the problems seen in cystic fibrosis. For example, a lower than normal phospholipase-C-coupled calcium response due to overexpression of NYD-SP27 in cystic fibrosis could render the calcium-dependent alternative secretory pathway ineffective, although calcium-activated chloride channels are known to be present in the pancreas and could be an alternative to the defective cAMP-dependent CFTR.16,17,18

The suppression of phospholipase-C-coupled protein-kinase-C activity by high concentrations of NYD-SP27 could still impair CFTR function, leading to pancreatic insufficiency in these patients. This idea may explain the recent findings of the analysis of exocrine pancreatic function in cystic fibrosis that mild CFTR mutation does not exclude pancreatic insufficiency.19 Therefore, abnormal expression of NYD-SP27 in cystic fibrosis could contribute to the pathogenesis of pancreatic insufficiency in most patients with cystic fibrosis. Although the cause for upregulation of NYD-SP27 in cystic fibrosis remains to be elucidated, CFTR is known to mediate transcription-factor activation and its mutation has resulted in altered gene expression of the chemokine RANTES.20 This study has also shown the possibility of circumventing defective pancreatic anion secretion in cystic fibrosis. Suppression of NYD-SP27 not only increased ATP-induced release of calcium ions and raised activity of protein kinase C, but also restored cAMP-dependent anion secretion in pancreatic-duct cells from patients with cystic fibrosis. The observed rescuing effect of NYD-SP27 on NYD-SP27-antiseense on cAMP-dependent pancreatic anion secretion was due mainly to rectification of CFTR by ATP and the ATP-potentiated forskolin-induced I_{sc} response by ATP was also shown. This finding could be due to increased ATP-induced activity of protein kinase C by suppression of NYD-SP27. The observation that the ATP-potentiated forskolin-induced I_{sc} response by SP27 was inhibited by the phospholipase-C inhibitor U73122 and protein-kinase-C inhibitor staurosporine suggests the involvement of phospholipase-C-coupled protein kinase C, supporting the notion that increased amounts of protein kinase C due to suppression of NYD-SP27 could potentiate the forskolin-induced I_{sc} response. Potentiation of cAMP responses by protein kinase C has also been observed in several CFTR-expressing tissues including pancreatic-duct cells.21 Protein kinase C increases both the rate and magnitude of subsequent protein-kinase-A stimulation of CFTR channel open probability.22 The lengthened plateau observed with the forskolin-stimulated I_{sc} in NYD-SP27-suppressed cells may reflect an increase in CFTR-channel open probability as a result of potentiation by high concentrations of protein kinase C. However, further investigation will be required to clarify the detailed mechanism.

The ability to restore pancreatic-duct anion secretion in NYD-SP27-suppressed cystic-fibrosis cells could provide a new possible alternative treatment for cystic fibrosis.

Contributors
Hu Zhu, Jianmin Li, Zuomin Zhou, and Jiahao Sha were responsible for gene identification. Jin Xia Zhu was responsible for gene identification and current measurements and confocal microscopy with assistance from Yi Yu and Jian Li Jiang did the calcium measurements. Hsiao Chang Chan, proof read by D K Rowlands, and approved by all the authors.Conflict of interest statement
None declared.

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References

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Clinical picture

Intracranial haemopoiesis

Thomas Kittner, Daniela Aust, Thomas Illmer

A 57-year-old woman had a 9-year history of polycythaemia vera with subsequent development of osteomyelofibrosis. She presented with malaise, dizziness, and diplopia. A T2-weighted MRI of the brain (figure) showed a large tumour mass in the area of the falx (arrows) with a finger-like perifocal oedema of the adjacent white matter. There was no evidence for a blastic transformation of the underlying disease. Emergency treatment with local radiation and intravenous dexamethasone was started. The patient’s neurological condition improved rapidly. However, her transfusion requirement increased and she was prepared for allogeneic stem-cell transplantation. During conditioning therapy the patient’s neurological condition deteriorated and she died with signs of disseminated encephalitis. Autopsy showed encephalitis. Moreover, we found a large tumour in the area of the falx cerebri. Histopathological examination showed intracranial haemopoiesis.